UNIVERSITY OF CALIFORNIA

Santa Barbara

The Effects of Invasive African Clawed Frogs on Native Amphibians in Southern California

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution, & Marine Biology

by

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June 2017

The dissertation of Emily Anne Wilson is approved.

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June 2017

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ACKNOWLEDGEMENTS

I would like to thank my committee members Cherie Briggs, Tom Dudley, and Steve Rothstein for their help and guidance throughout my research. I give a special thanks to Cherie and Tom who welcomed me to their labs in a particularly challenging transition during my graduate career. You have always been there for me whenever I asked for advice and your support has allowed me to complete this degree.

I owe a debt of gratitude to Renwei Chen and Mary Toothman who acted as my guides in the lab. They devoted many hours to train me on molecular techniques and were there to help troubleshoot when trouble inevitably arose. I am now an expert in manipulating small volumes of liquids and detecting invisible DNA sequences in those liquids, thanks to them. Basically, they made me a magician.

I thank my additional collaborators Ed Ervin and Panu Somervuo. Ed was kind enough to join me in the field and shared his knowledge of African clawed frog habits and population sites in southern California, which was invaluable. Our days of slogging through southern California waterways, seining for frogs, were later replaced by collecting water samples to detect their presence using environmental DNA. Panu, the bioinformatician guru, waded through the gigabites of next-generation sequencing data from those eDNA samples to find the useful amphibian sequences.

Others who assisted in this work include: Scott Cooper who advised me on and reviewed the behavioral experiments; Chris Jerde, Kristy Deiner, and Caren Goldberg who helped me break into the world of environmental DNA research; Steve Howard and Mike Booth of United Water, who provided access to field sites and helped me collect specimens; Sandy Hedrick of Hedrick Ranch Nature Area, and Friends of the Santa Clara River who gave me access to an African clawed frog site and provided a location to perform field experiments. I could not have done this without you.

Financial support was provided by several generous organizations and supporters at UCSB: Santa Barbara Coastal Fund grants (WIN13-13; FALL 14-29); the Department of Ecology, Evolution, & Marine Biology Block Grant for summer research; UCSB STEM Student Support Fund; Susan & Bruce Worster Internship and Mentorship Award; Mellichamp Fellowship for Graduate Students in Systems Biology & Bioengineering.

I am grateful to the team of undergraduates helped me throughout my research: Hunter McCrea, Lourdes Velazquez, Rana Mirzaie, Taylor Johnson, James Thomson, Leah Arth, An Bui, Annie Rosen, Fiona Chan, Isabel Wang, and Tori Prado. Their devotion in the field, reviewing hours of tadpole videos, caring for the animals in lab, and bleaching an incredible amount of equipment, cannot be understated.

My family and friends were always there for support when needed. I want to thank my parents who encouraged me to pursue my love of amphibians into graduate school. I am thankful for my family and friends who were provided sound advice and good company along the long road to my degree.

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Wilson, E., T. Dudley, & C. Briggs. 2016. Feral *Xenopus laevis*: a source or sink for Bd? Poster presentation. California & Nevada Amphibian Populations Task Force Annual Meeting, Tempe, AZ.

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ABSTRACT

The Effects of Invasive African Clawed Frogs on Native Amphibians in Southern California

by

Emily Anne Wilson

With increased global trade and human movement, invasive species have established populations in new regions at an unnaturally high rate, threatening native species and ecosystems. One invasive amphibian, the African clawed frog (*Xenopus laevis*), native to sub-Saharan Africa and shipped globally beginning in the mid 20th century, has established populations across the world, including southern California. Little research has been performed to determine how this invasive species may affect native amphibian populations already at risk from other anthropogenic factors.

The first part of this dissertation explores how *X. laevis* affects the abundance of the amphibian chytrid pathogen, *Batrachochytrium dendrobatidis* (Bd). As an asymptomatic carrier of Bd, post-metamorphic *X. laevis* were hypothesized to be large reservoirs of the pathogen, responsible for spreading it globally to susceptible native species. However, field surveys from three populations of *X. laevis* found few infected individuals and those that were infected had low infection levels. Laboratory experiments were performed to evaluate the potential for larval *X. laevis* to prey upon the motile infectious zoospore stage of the pathogen in the water column. A reduction in the motile zoospores has been associated with reduced transmission rates and larval *X. laevis* in laboratory experiments was found to consume live Bd zoospores. However, larval *X. laevis* also exhibited intraguild predation on

a zooplankton, *Daphnia magna*, which itself preys upon the chytrid zoospores. Intraguild predation may complicate the net effect of *X. laevis* larvae on Bd zoospore abundance in the water column. Together, these findings suggest that *X. laevis* is likely not a large source of the pathogen and may prey upon Bd, suppressing pathogen transmission to or between native amphibians.

The second part of this dissertation explores *X. laevis* as a predatory threat to amphibians native to southern California, and native amphibian response to the potential predation threat. Laboratory predation trials were performed using the Pacific treefrog (*Pseudacris regilla*) as a representative of native California amphibians, and showed that *X. laevis* will prey upon larval, juvenile, and adult *P. regilla*. Behavioral trials were also performed to evaluate if *P. regilla* larvae and adults recognize *X. laevis* as a potential predator and alter their behavior to avoid predation. These experiments found that while *P. regilla* larvae do not change their activity levels in the presence of *X. laevis*, they do display spatially avoidance. Field enclosure experiments with adult *P. regilla* also found that they will spatially avoid *X. laevis*. This suggests native amphibians may recognize *X. laevis* as a predator, invoking a spatial avoidance response.

The third part of this dissertation explores the distribution of *X. laevis* and its co-occurrence with native amphibians to determine if the spatial avoidance observed in the experimental trials with *P. regilla* translates into the exclusion of amphibian populations from their native habitat. Amphibian surveys were performed using a new molecular technique, environmental DNA, to detect species' presence through DNA shed into the water. Environmental DNA successful detected the presence of *X. laevis* using a *Xenopus*-specific primer with quantitative PCR, further establishing this method as a useful tool to survey *X. laevis* distribution. Another more general primer was used to detect all amphibian

species and explore co-occurrence between *X. laevis* and native amphibians. Several species of native amphibians were present at the same stream sites as *X. laevis* in locations throughout southern California.

Together this dissertation suggests that while *X. laevis* is not acting as an ecologically important reservoir for the chytrid pathogen, it is a threat to native amphibians because of direct predation on larval and adult stages. Native amphibians may recognize and avoid *X. laevis*, reducing predation risk; but potentially reducing native amphibians in areas invaded by *X. laevis*. Survey for *X. laevis* revealed its widespread presence in southern California and co-occurrence with multiple native amphibian species.

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Chapter 1

Invasive African Clawed Frogs in California: A Reservoir or Control for *Batrachochytrium dendrobatidis*?

Abstract

Amphibian species are experiencing population declines due to infection by the amphibian pathogen, *Batrachochytrium dendrobatidis* (Bd). The African clawed frog (*Xenopus laevis*) is an asymptomatic carrier of Bd and has been implicated in the spread of this pathogen through global trade. This species has established invasive populations on several continents and the post-metamorphic individuals may act as a reservoir, spreading the infection to susceptible species, while the larvae are filter-feeders that potentially consume the motile Bd zoospores from the water column, which may reduce pathogen abundances and thus the likelihood of infection. We evaluated the importance of these contrasting processes in southern California by testing populations of post-metamorphic individuals for infection and we performed laboratory experiments to determine if larval *X. laevis* preyed upon Bd zoospores. Water fleas (*Daphnia magna*) were also included in the Bd consumption trials to compare consumption rates and also in later predation trials to establish whether intraguild predation between the larval *X. laevis* and *D. magna* may occur, potentially interfering with control of Bd zoospores by *D. magna*. The field surveys of three sites tested 70 post-metamorphic individual *X. laevis* for Bd found a 10% infection prevalence and all infection loads below 5 zoospore equivalents, which is considered to be a low and sublethal Bd load in most amphibian species. Laboratory experiments found that larval *X. laevis* consume Bd zoospores and therefore may function as a control for Bd, reducing transmission between amphibians. However, metamorphic and juvenile *X. laevis* exhibited intraguild predation by consuming *D. magna*, which also prey upon Bd zoospores. The results suggest that *X laevis* is not a large reservoir for Bd and its larval stage may offer some control for Bd transmission.

Introduction

The amphibian pathogen, *Batrachochytrium dendrobatidis* (Bd), is responsible for population declines and extinctions of many amphibian species worldwide (Stuart, 2006; Vredenburg et al., 2010). Understanding the transmission of the pathogen is complicated by its uneven impacts across species. While some species experience rapid mortality when infected, others are asymptomatic carriers with no negative effects from the infection (Briggs et al., 2010). Species vary in their potential to transmit the pathogen to other organisms, in that some species or life stages can act as a reservoir, harboring the pathogen that subsequently infect other amphibians (McMahon et al., 2013; Reeder et al., 2012), while other species may exhibit behaviors that reduce the abundances or infective potential of the pathogen, which reduces transmission potential (Buck et al., 2011; Searle et al., 2013).

Reservoir species are infected carriers, often displaying few symptoms of infection (Mandl et al., 2015). These carrier species can be detrimental to susceptible symptomatic species by facilitating pathogen retention in an environment following the extirpation of susceptible amphibian species. As a result, the ability of the populations to rebound following an initial pathogen driven die-off may become more difficult for susceptible species (Reed et al. 2012). Many species have been found to carry Bd and may act as reservoirs, spreading the pathogen to susceptible amphibians. Two invasive amphibian species, the African clawed frog (*Xenopus laevis*) and American bullfrog (*Lithobates catesbeianus*), are likely Bd reservoirs and are often implicated as spreading the pathogen globally (Garner et al., 2006; Hanselmann et al., 2004; Weldon et al., 2004). North American crayfish species (*Procambarus spp*. and *Orconectes virilis*) can also harbor and transmit Bd infection and given their widespread invasion into waterways, could be a important vector of Bd (McMahon et al., 2013). Native species can also act as fungal

reservoirs. Amphibians such as the Pacific treefrog (*Pseudacris regilla*) can sustain an infection with little evidence of disease but may spread the pathogen as they move across a landscape (Reeder et al., 2012). Populations of susceptible species are less likely to rebound if a reservoir species is still present in the area because exposure to the asymptomatic reservoir carrier species can cause another outbreak and population crash (McMahon et al., 2013).

Conversely, within the aquatic environment, consumers capable of feeding on infective agents can potentially function as biological controls of Bd population size. Zooplankton, such as *Daphnia* (Buck et al., 2011; Hamilton et al., 2012; Searle et al., 2013) and ciliates (Schmeller et al., 2014) consume the motile zoospore stage of Bd from the water column. The reduction of Bd zoospores may lead to reduced transmission rates between amphibians in the water (Schmeller et al., 2014; Searle et al., 2013).

Xenopus laevis is unique among potential reservoir species because it may function as both a reservoir and a control for the pathogen, depending on its life stage. Adult *X. laevis* are asymptomatic carriers of Bd (Ramsey et al., 2010). The species is fully aquatic and could expose native amphibians to the infectious stage of Bd if they share water sources. Bd transmission occurs through a motile zoospore stage that swims through the water to infect a new host or re-infect the current host. The higher the infection intensity an individual has, the larger the zoospore output and thus the higher potential for transmission (DiRenzo et al., 2014). To understand the impacts of the infection it is necessary to measure the proportion of infected individuals in a population, referred to as the 'prevalence,' and the intensity of infection per individual, the infection 'load.'

Bd infection prevalence and loads vary regionally in *X. laevis* populations (Table 1.1). In its sub-Saharan African range, *X. laevis* infection prevalence range from 25.2%

(Weldon, 2005) to 0.25% (Vredenburg et al., 2013; Weldon et al., 2004). In its introduced range, *X. laevis* in Wales, UK displayed the greatest variation in Bd infection prevalence between 0% and 83.6%, and mean infection loads ranged from 38.9 and 1,295.9 genetic equivalents (GE), depending on location and season (Tinsley et al., 2015). In Chile, only three out of ten sites with *X. laevis* were Bd positive, all with low infection loads; however, the overall infection prevalence was 24% (Solís et al., 2009). In France, a population was surveyed for Bd but none of the specimens was positive (Ouellet et al., 2012) and in Japan a population was estimated to have an infection prevalence of 13% (Goka et al., 2009). In California, *X. laevis* infection levels and prevalence have been estimated only from preserved specimens. One study of museum specimens estimated prevalence at 13% and all infection loads were less than one GE (Vredenburg et al., 2013). A separate analysis found that previously collected specimens estimated infection prevalence at 4% in California (Weldon, 2005).

With such variation in *X. laevis* infection prevalence and loads globally, it is difficult to say with certainty what infection levels *X. laevis* populations will have in a particular region. There appears to be potential for invasive *X. laevis* populations to act as a reservoir for Bd, capable of driving a Bd outbreak in an area where individuals are harboring high loads or have high prevalence of infection (Solís et al., 2009; Tinsley et al., 2015). There are also populations of *X. laevis* that either do not harbor Bd or have low infection prevalence and loads (Ouellet et al., 2012; Weldon, 2005). Many of these specimens were collected decades ago, adding additional uncertainty concerning the current status of *X. laevis* infection so an update evaluation of *X. laevis* infection levels is needed to determine is current status as a potential reservoir in southern California.

In addition to potentially acting as a current reservoir, a research avenue that has not yet been explored is the potential for *X. laevis* to also act as a control agent for Bd. As obligate filter feeders, *X. laevis* larvae could function as a Bd predator by consuming Bd zoospores from the water column. Zoospores average of 3-5 μm in diameter (Longcore et al., 1999) and larval *X. laevis* are capable of removing particles from 0.2 μm to over 200 μm from the water (Seale et al., 1982). Larval *X. laevis* cannot, however, be a source of Bd because the zoospores infect only the keratinized structures found in the skin of post-metamorphic amphibians and the mouthparts of larval anurans (Voyles et al., 2011). As filter feeding specialists, *X. laevis* larvae lack keratinized tooth-like mouthparts used for grazing (Wassersug, 1996) and therefore cannot harbor a Bd infection. Only when the *X. laevis* metamorphose do they produce keratinized structures in their skin that are susceptible to infection.

Larval *X. laevis* have the potential to be more effective at removing Bd from the water column than zooplankton. Zooplankton are a fraction the size of *X. laevis* larvae so their filtration rates cannot rival a *X. laevis* larva but zooplankton make have greater densities to compensate for their smaller size. *X. laevis* may also have a lower particle threshold than zooplankton, making them capable of feeding at lower particle concentrations (Seale et al., 1982). Zooplankton could compensate for their smaller size, however, by their potential to occur at higher densities than *X. laevis* in aquatic communities. To evaluate how effective *X. laevis* larvae are we compared their Bd zoospore consumption rate to that of a zooplankton species, *Daphnia magna*.

The impact of larval *X. laevis* as a predator suppressing Bd zoospore abundances may be offset by the potential for *X. laevis* larvae and juveniles to act as intraguild predators on native zooplankton that also feed on Bd. Zooplankton such as *Daphnia* have been suggested

as a potential controls for Bd (Buck et al., 2011; Schmeller et al., 2014) so any significant loss in natural zooplankton populations could negate positive effects of predation by *X. laevis* larvae on Bd. Larval *X. laevis* are known to filter small prey particles out of the water column and transition to larger zooplankton as they metamorphose and develop into juveniles (Schoonbee et al., 1992). It is therefore necessary to determine the developmental stage *X. laevis* could become an intraguild predator by consuming the relatively large zooplankton such as *D. magna.*

This study tests whether *X. laevis* act as a reservoir or a control for Bd in southern California. To test whether *X. laevis* are a reservoir for Bd, post-metamorphic *X. laevis* wild populations in southern California were surveyed and tested for Bd infection to determine prevalence and load of infection. To test whether larval *X. laevis* could act as a control for Bd, laboratory experiments were performed determine if larval *X. laevis* consume Bd zoospores from the water column and if those consumption rates are comparable to *D. magna*. Laboratory predation trial with *X. laevis* larvae and juveniles were performed to determine at what developmental stage *X. laevis* become capable of consuming large zooplankton that also feed on Bd zoospores.

Methods

Wild *X. laevis* Bd infection levels

Individual *X. laevis* were captured in the field and evaluated for Bd infection prevalence and load. The *X. laevis* were collected from 3 sites: an isolated pond on Hedrick Ranch Nature Area, adjacent to the Santa Clara River, Ventura County; isolated pools on Piru Creek, Ventura County; and Murray Canyon Creek, San Diego County. Any other

amphibian species present at the sites were included in the study when found but other species were not specifically targeted. Individuals were captured by funnel minnow trap, seine or dipnet. Individuals were handled with clean gloves and their ventral surfaces were swabbed with a sterile cotton-tip swab following the protocol of Hyatt et al. (2007) to collect Bd cells for genetic detection. Swabs were either field dried and stored at room temperature or if not dried, stored at -4°C.

Swabs were processed in triplicate using a quantitative PCR assay following the protocol of Boyle et al. (Boyle et al., 2004) with Life Technologies Taqman Universal Master Mix or Bioline Sensifast Master Mix. Amplification standards of 0.1, 1, 10, 100, and 1,000 zoospore equivalents, isolated from 60 Lakes Basin, Kings Canyon National Park in 2009, were included in each assay to quantify the amount of Bd on each swab. An individual was considered infected if a single replicate was positive for Bd. An individual's Bd infection load was calculated by averaging all positive quantitative PCR results from the individual's three replicates.

Larval *X. laevis* & *D. magna* consumption of Bd

A laboratory experiment was performed to determine if larval *X. laevis* consume Bd zoospores, and if so, how their consumption rates compare to adult *D. magna*. The larval *X. laevis* were purchased commercially (Nasco®), fed Nasco Frog Brittle powder *ad libitum*, and then fasted 24 hours prior to the experiment. The *D. magna* were purchased commercially (Ward's Scientific), fed yeast powder *ad libitum*, and then fasted 24 hours prior to the experiment.

The experiment was performed in 400 mL plastic cups filled with 120 mL of purified bottled water. One *X. laevis* larva or three adult *D. magna* were placed in one of the six

treatments: Bd present with a live *X. laevis* larva (n=16); Bd present with a dead *X. laevis* larva (n=8); Bd present with live *D. magna* (n=8); Bd present with dead *D. magna* (n=4); Bd absent with a live *X. laevis* larva (n=5); Bd absent with live *D. magna* (n=3).

The treatments that exposed dead *X. laevis* or dead *D. magna* to Bd were included in the experiment to distinguish actively consumed Bd zoospores from any zoospores that might inadvertently swim in the mouth of a *X. laevis* or attach to the carapace of the *D. magna*. The larval *X. laevis* were euthanized in a buffered MS222 solution (5g/L) for one hour and the adult *D. magna* were euthanized in 70% ethanol. The euthanized animals were then rinsed twice with fresh water before placement into the experiment.

The Bd was cultured in the laboratory from the CJB7 isolate collected from Sixty Lake Basin in Kings Canyon National Park, California. The concentration of Bd zoospores was counted using a hemocytometer. Each treatment containing Bd was inoculated with 442,000 zoospores.

The experiment ran for 4.5 hours, after which all animals were removed from the treatments and rinsed thoroughly with fresh water. Live *X. laevis* and *D. magna* were immediately euthanized with MS222 or ethanol, respectively. All animals were then preserved in ethanol. The gut of each *X. laevis* was dissected from esophagus to vent and cut into pieces. The *D. magna* in each treatment were pulverized with a 1.5mL vial pestle in preparation for DNA extraction. DNA extraction was performed using the Qiagen DNeasy Blood & Tissue Kit and protocol with the exception that the tissues were incubated overnight in the lysis step to facilitate complete tissue breakdown. Each sample was analyzed in duplicate using the same quantitative PCR protocol described in the "Wild *X. laevis* Bd infection levels" section. The Bd results for each treatment were averaged.

Larval *X. laevis* predation on *D. magna*

A second laboratory experiment was performed to measure *X. laevis* predation on *D. magna*. The animals were procured from the same commercial suppliers and had the same diet as those animals used in the previous experiment, except that the juvenile *X. laevis* were fed small pellets of Nasco Frog Brittle. The zooplankton, *D. magna*, were divided into three separate classes based on size measured from the crown of the head to the base of the spine: neonates (≤ 1 mm), juveniles (< 1 to 2.25 mm); adults (≥ 2.25 mm) (Barata and Baird, 1998; Green, 1956).

Five *D. magna* of the same size class were placed in 400 mL cups filled with 200 mL of purified bottle water. A single larval (Gosner stages 26-42), metamorphic (Gosner stages 45-46), or juvenile (SVL 24-30 mm) *X. laevis* was fasted for 24 hours prior to the experiment and placed in a cup with *D. magna* (Gosner, 1960). The experiment was performed in the following factorial design: a larval *X. laevis* with four replicates of each *D. magna* size class (n=12); a metamorphic *X. laevis* with two replicates of each *D. magna* class (n=6); and a juvenile *X. laevis* with two replicates of each *D. magna* class (n=6). The *X. laevis* were given 24 hours to consume the *D. magna*, after which any remaining *D. magna* were counted.

Results

Wild *X. laevis* Bd infection levels

A total of 70 *X. laevis* were collected at the three sites between November 2012 and May 2015: 31 from the Hedrick Ranch Nature Area (HRNA) pond from four separate visits between November 2012 and May 2014; nine from one visit to Murray Canyon Creek in March 2014; and 30 from five separate visits to Piru Creek pools between May 2014 and

2015. Seven of the *X. laevis* were found to be positive for Bd; two from HRNA (2.6; 4.6 Zoospore Equivalents), one from Murray Canyon (0.32 ZE), and four from Piru Creek (<0.1; 3.4; 3.9; 4.4 ZE) (Table 1.2). For a complete list of the collection locations, dates, and results see Appendix 1.1.

Four American bullfrogs (*Lithobates catesbeianus*) were also captured at the Piru Creek pools on one site visit and were included in the study. The Piru Creek site is a series of three pools all within approximately 200 yards of each other where the majority of the *X. laevis* were collected from the southernmost pool and the majority of the *L. catesbeianus* were collected from the larger northernmost pool. The species co-occurred in the middle pool. The *L. catesbeianus* were collected during an invasive species removal project that coincided with visits to this site. All four *L. catesbeianus* individuals were Bd positive (1.3; 7.0; 9.6; 421.2 ZE).

Larval *X. laevis* & *D. magna* consumption of Bd

Both the larval *X. laevis* and the adult *D. magna* consumed Bd zoospores. A permutation ANOVA analysis was performed on the quantitative PCR results to determine the number of zoospores actively consumed by the *X. laevis* and *D. magna*. The analysis found significant differences between the four treatments tested: live *X. laevis*, live *D. magna*, dead *X. laevis*, and dead *D. magna* (Permutation ANOVA: DF=3, iterations=5,000, p=<0.01). Each of the four treatments was significantly different (FDR p-value adjustment, p<0.05) (Figure 1.1). Larval *X. laevis* consumed significantly more Bd zoospores, an average of 11,547 ZE (± 6,545 SE, n=16), while the sets of three adult *D. magna* consumed an average of 619 ZE (± 68.2 SE, n=8), as measured by quantitative PCR of the *X. laevis* guts or *D. magna* bodies. Only trace amount of Bd (0.004 ZE ± 0.001 SE, n=8) were found in the

guts of the dead *X. laevis* that were exposed to Bd zoospores. Larger numbers of Bd zoospores were found in the dead *D. magna* (25.6 ZE ± 6.26 SE, n=4), likely from zoospores attached to the outer carapace of the crustacean since the entire organism was included in the DNA extraction, rather than only the gut. If the average number of zoospores attached to dead *D. magna* is subtracted from the set of three *D. magna*, the average becomes approximately 591 ZE, or 197 ZE for each individual *D. magna*. Zoospores were largely absent on *X. laevis* larvae (n=5) and *D. magna* (n=3) in the negative Bd controls, averaging fewer than one for each consumer.

There was no significant relationship between the developmental stage of *X. laevis* larvae and the number of zoospores an individual consumed (R^2=0.13, $F_{1,14}$=2.159, p>0.05) (Figure 1.2).

Larval *X. laevis* predation on *D. magna*

Larval *X. laevis* (Gosner 26-42) did not consume any *D. magna* but metamorphic (Gosner 45-46) and juvenile *X. laevis* consumed all individuals of all size classes of this crustacean (Table 1.3). In the treatments with larval *X. laevis*, two dead *D. magna* were present but not consumed.

The larval *X. laevis* appeared to actively avoid *D. magna* while continuously filtering water. The two species were often in close proximity but the larvae were never observed actively moving towards, chasing, or otherwise attempting to capture the *D. magna*. The *X. laevis* metamorphs and juveniles were not observed filter feeding and appeared to quickly detect the *D. magna*. They would orient towards the zooplankter and quickly capture it in a lunging motion.

Discussion

The results do not support the hypothesis that *X. laevis* are reservoirs of Bd in southern California based on the results from the Bd swabs. The three invasive populations of *X. laevis* surveyed in this study had a low prevalence of Bd (10%) and a maximum infection load of 4.6 zoospore equivalents (ZE). The combination of low prevalence and low loads suggest it is unlikely that *X. laevis* is driving transmission or infection of the pathogen among susceptible amphibians.

The low prevalence and infection load values in this study are comparable to the results from the *X. laevis* museum specimens collected across California, 13% Bd prevalence and loads less than one genetic equivalent (Vredenburg et al., 2013). It should be noted, however, that qPCR analysis of museum collections will likely underestimate Bd presence and loads because of the degradation of Bd DNA from the museum preservation methods that use formalin, particularly on specimens with low infection levels (Adams et al., 2015). It is therefore possible that these *X. laevis* museum specimens collected in previous decades had higher Bd prevalence and loads. The findings of this study are only slightly higher than histological detection of Bd on museum specimens from California that found 4% of the *X. laevis* infected with Bd (Weldon, 2005). The consistently low prevalence from these two previous studies and the live capture specimens from this study suggests that *X. laevis* have not been a large reservoir for Bd in California in the past decades nor are they currently.

In our study, animals were collected over several months and years, which may have captured Bd infection variation that appeared to be present in the *X. laevis* populations in other regions (Tinsley et al., 2015). Attempts were made to include more populations in this study since the Solís et al. (2010) study found Bd positive individuals in only three out of

their ten sites but the drought in southern California over the study period reduced availability of habitats suitable for *X. laevis*.

One population of *L. catesbeianus* in the series of pools at the Piru Creek site, which co-occurred with some of the *X. laevis* included in this study, had higher Bd prevalence and loads than the *X. laevis* tested at that site. While this was a small sample size of *L. catesbeianus* (n=4), the high prevalence and loads are consistent with studies from other regions (Garner et al., 2006; Pearl et al., 2007; Yang et al., 2009). Despite the higher infection prevalence and loads on the *L. catesbeianus* at Piru Creek, the *X. laevis* at this site had a 13% infection prevalence and all Bd loads were less than five ZE. This suggests that other amphibians, such as *L. catesbeianus*, may be greater reservoir of Bd than *X. laevis*.

In the laboratory, larval *X. laevis* were capable of consuming Bd zoospores from the water column and thereby may act as a controlling mechanism for Bd. By consuming infectious zoospores, *X. laevis* could potentially reduce Bd abundances leading to lower probability of transmission between amphibians, as has been shown with zooplankton feeding on zoospores in laboratory trials (Hamilton et al., 2012; Schmeller et al., 2014; Searle et al., 2013; Venesky et al., 2013). Larval *X. laevis* consumed a large number of Bd zoospores, an average of over 10,000 zoospores per individual in the 4.5 hour trial while a single *D. magna* consumed an average of almost 200 zoospores. With these estimates, it would take over 50 *D. magna* to consume the same number of Bd zoospores as one *X. laevis* larva. The *D. magna* adult and *X. laevis* larva consumption rates serve as estimate that do not take into account changes in consumption over time and under different Bd concentrations. But *Daphnia* are discriminate predators and preferentially select prey based on size and structure (DeMott, 1995). Any ability to seek out Bd zoospores could make them more

efficient predators than the generalist filter feeding *X. laevis*, particularly if Bd zoospores are a sought after prey item and were at low concentrations.

The number of Bd zoospores consumed did not vary with *X. laevis* larval developmental stage. We expected to observe higher Bd consumption among the largest larvae because of greater size and therefore pumping volumes. It is unclear whatcaused the lack of a relationship between larval size and Bd consumption. *X. laevis* larvae are known to adjust their pumping rate to regulate their ingestion rate at different food particle concentrations (Seale et al., 1982), so the size of a larvae may not be an indicator filter rate.

Since both larval *X. laevis* and adult *D. magna* consume Bd, intraguild predation could further complicate the control of Bd. Our results confirm previous work indicating that larval *X. laevis* consume small food items such as phytoplankton, and transition to consuming zooplankton during metamorphosis (Schoonbee et al., 1992). Only when the *X. laevis* were metamorphosing would they consume *D. magna*, even though larvae have mouths wide enough that gape limitation should not occur with adult *D. magna*. If *X. laevis* consume large numbers of *Daphnia*, they could interfere with control of Bd by *D. magna* or other zooplankton the *X. laevis* prey upon.

Daphnia are only one of the potential zooplankton predators of Bd. Ciliates and rotifers also consume Bd and research suggests they can reduce the transmission of Bd between amphibians (Schmeller et al., 2014). Rotifers and ciliates have a wide range of sizes and some are less than 200 μm (Snell and Carrillo, 1984), within the filtration particle size of larval *X. laevis* (Seale et al. 1982). The size range of zooplankton could be associated with substantial reduction in zooplankton abundance in the presence of *X. laevis*, if larval *X. laevis* consume the smaller ciliates and rotifers size classes, while the metamorphosing and metamorphic *X. laevis* consume the larger.

This study suggests that invasive populations of *X. laevis* do not act as major reservoir of Bd infections in southern California. While we found that *X. laevis* larvae can consume Bd zoospores, they are unlikely to be an effective agent of biological control for Bd. Metamorphic *X. laevis* consume native zooplankton that may serve as a better predator to Bd zoospores. Zooplankton, such as *Daphnia* and ciliates, could reach high enough densities in the environment to reduce Bd zoospore concentrations and Bd transmission. Zooplankton are also more suitable regulators of Bd abundance than are *X. laevis* larvae because they are native to the ecosystem and are not likely to displace any other native species. Invasive *X. laevis* negatively affect native amphibians and aquatic invertebrates through predation and native amphibian displacement (Amaral and Rebelo, 2012; Lillo et al., 2011), that make them undesirable even if they do not pose a risk as reservoirs of Bd.

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Tables & Figures

Author	Region	Specimen Type	Detection Method	Number of Specimens	Prevalence	Load (GE)
Weldon et al. 2005	South Africa	Live Capture	Histology - toe webbing	365	25.2%	N/A
Weldon et al. 2004	Africa	Museum Collection	Histology - toe webbing	583	2.6%	N/A
Soto-Azat et al. 2010	Africa	Museum Collection	qPCR - swab	249	1.2%	≤ 10.3
Vredenberg et al. 2013	Africa	Museum Collection	qPCR - swab	122	0.25%	≤ 2
Solis et al. 2010	Chile	Live Capture	qPCR - toe clip	58	24%	≤ 10
Tinsley et al. 2015	Wales, UK	Live Capture	qPCR - swab	253	0-88%	up to ~13,000
Ouellet et al. 2012	France	Live Capture	Histology - toe clip	89	0%	N/A
Goka et al. 2009	Japan	Live Capture	PCR - swab	168	13%	N/A
Vredenberg et al. 2013	California	Museum Collection	qPCR - swab	23	13%	≤ 1
Weldon et al. 2005	California	Museum Collection	Histology - toe webbing	102	4%	N/A

Table 1.1. Literature on chytrid infection in wild *X. laevis* populations
Previously published results of Bd infection prevalence and load found in both native and invasive *X. laevis* populations.

Site	Number of *X. laevis* Tested	Prevalence	Average Load (ZE)
HRNA	31	6.5%	3.6
Murray Canyon	9	11.1%	0.3
Piru Creek	30	13.3%	2.7

Table 1.2. Chytrid infection in *X. laevis* populations in southern California
Bd infection prevalence and load (zoospore equivalents) from live *X. laevis* collected from invasive populations in southern California. Collection dates ranged between 2012 and 2015.

	D. magna		
	Neonates	**Juveniles**	**Adults**
***X. laevis* larvae (n=12)**	0/20	0/20 (1 dead)	0/20 (1 dead)
***X. laevis* metamorphs (n=6)**	10/10	10/10	10/10
***X. laevis* juveniles (n=6)**	10/10	10/10	10/10

Table 1.3. *X. laevis* predation on *D. magna*
Number of *D. magna* of different life stages consumed by larval, metamorphic, or juvenile *X. laevis*.

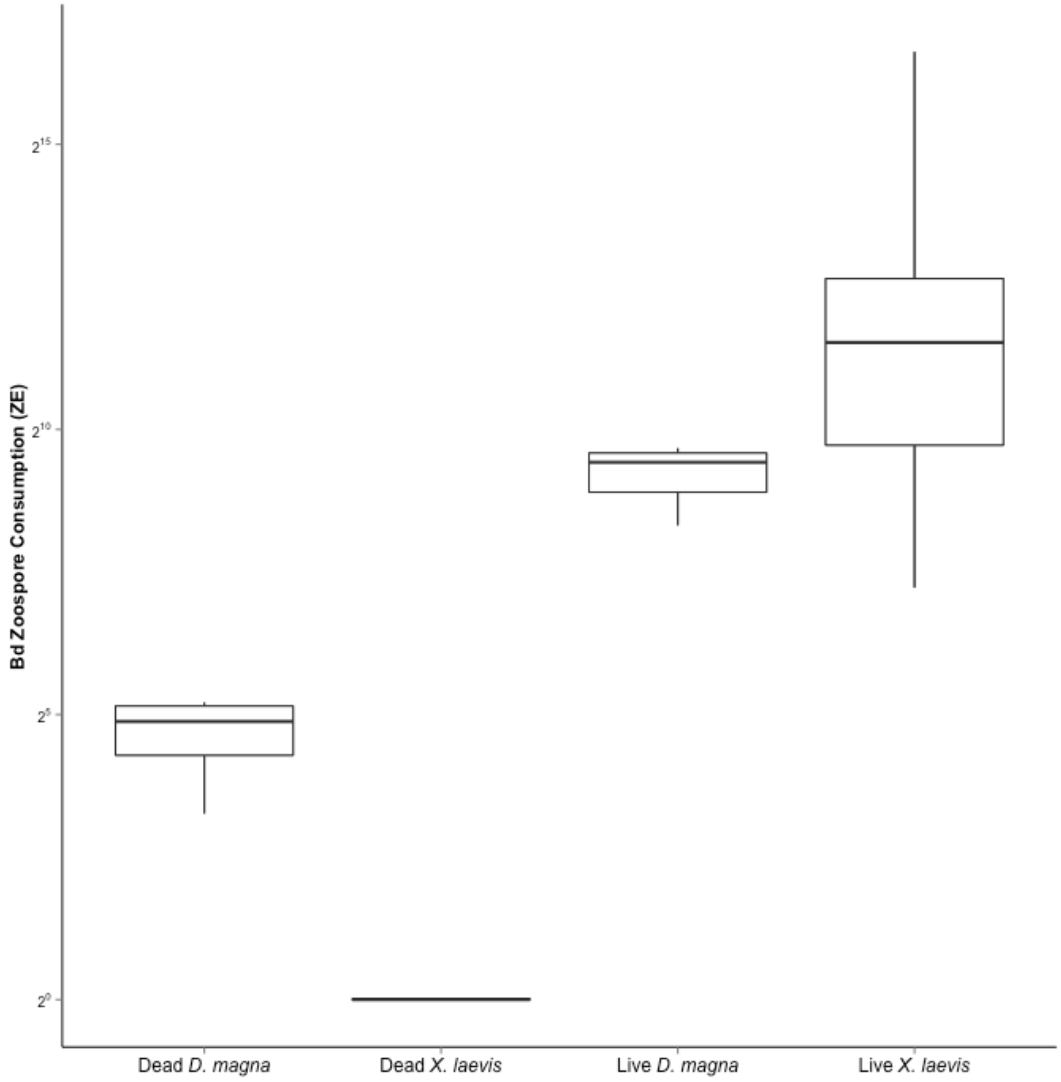

Figure 1.1. Bd zoospores consumed by larval *X. laevis* and *D. magna*
Average number of Bd zoospores found in the guts of *X. laevis* or on/in the entire three *D. magna* after 4.5 hours of exposure to 442,000 zoospores. The zoospore values were log transformed to normalize the range of Bd zoospore values. All treatment groups were significantly different from each other (Permutation ANOVA: DF=3, iterations=5,000, p=<0.01; FDR p-value adjustments, p<0.05)

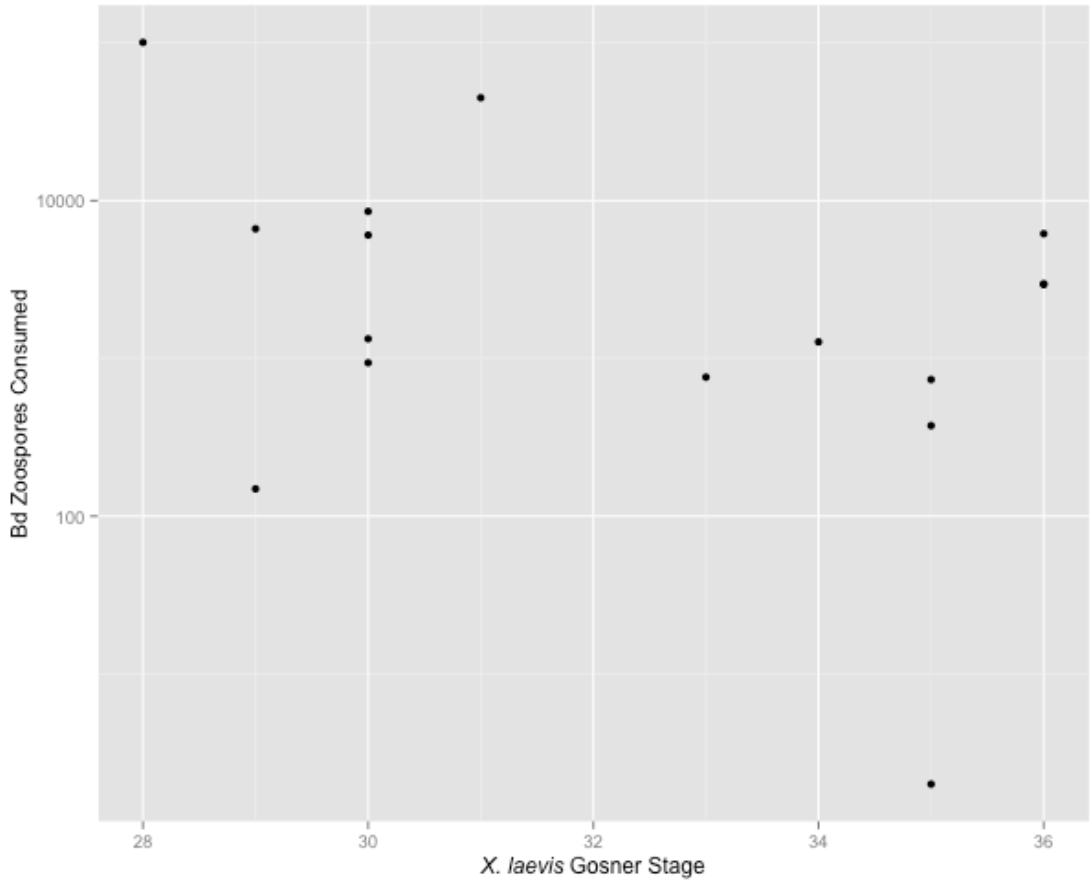

Figure 1.2. Larval *X. laevis* consumption of Bd zoospores by developmental stage
Number of zoospores consumed by *X. laevis* larvae of varying developmental stages when exposed to 442,000 zoospores over 4.5 hours. Bd zoospores are calculated in zoospore equivalents.

Appendix 1.1. Individual *X. laevis* results from assay for Bd infection

Xenopus laevis collection locations, dates, and results from the quantitative PCR detection of Bd infection. Each individual *X. laevis* sample was run in triplicate and any positives averaged to create the average Bd load in zoospore equivalents (ZE).

Site	Sample Date	Run 1 Load (ZE)	Run 2 Load (ZE)	Run 3 Load (ZE)	Average Load (ZE)
Hedrick Ranch Nature Area, Ventura County	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	18-Nov-12	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	10-Mar-13	0	0	0	**0**
	17-Apr-13	0	0	0	**0**
	17-Apr-13	0	0	0	**0**
	17-Apr-13	0.46	0	8.71	**4.59**
	17-Apr-13	0	0	0	**0**
	17-Apr-13	0	0	0	**0**
	17-Apr-13	0	0	0	**0**
	17-Apr-13	0.96	2.81	4.08	**2.62**
	11-May-14	0	0	0	**0**
	11-May-14	0	0	0	**0**
	11-May-14	0	0	0	**0**
	11-May-14	0	0	0	**0**
	11-May-14	0	0	0	**0**
Murray Canyon Creek, San Diego County	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0.32	**0.32**

	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0	**0**
	22-Mar-14	0	0	0	**0**
Piru Creek, Ventura County	6-May-14	0	0	0	**0**
	6-May-14	0	0	0	**0**
	6-May-14	0	0	0	**0**
	6-May-14	0	0	0	**0**
	6-May-14	0	0	0	**0**
	8-May-14	0	0	0	**0**
	8-May-14	0	0	0	**0**
	8-May-14	0	0	0	**0**
	8-May-14	0	0	0	**0**
	8-May-14	0	0	0	**0**
	18-Jun-14	1.61×10^{-4}	0	0	1.61×10^{-4}
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	18-Jun-14	0	0	0	**0**
	26-May-15	0	0	0	**0**
	26-May-15	0	0	3.90	**3.90**
	26-May-15	0	0	0	**0**
	26-May-15	0	0	4.36	**4.36**
	26-May-15	0	0	0	**0**
	26-May-15	0	0	0	**0**
	26-May-15	0	0	2.38	**2.38**
	26-May-15	0	0	0	**0**

Chapter 2

Share Behavioral Responses and Predation Risk of a California Anuran Larvae and Adults when Exposed to African Clawed Frogs

Abstract

Invasive species are a regional and global threat to biological diversity. In order to evaluate an invasive predator species' potential to harm populations of native prey species, it is critical to evaluate the behavioral responses of all life stages of the native prey species to the novel predator. The invasion of the African clawed frog (*Xenopus laevis*) into southern California provides an opportunity to evaluate the predation risk and behavioral responses of native amphibians. We performed predation trials and explored prey behavioral responses to determine how this invasive predator may impact native amphibian populations using Pacific chorus frogs (*Pseudacris regilla*) as a representative native California prey species. We found that *X. laevis* will readily prey upon larval and adult life stages of *P. regilla*. Behavior trials indicated that both larval and adult *P. regilla* exhibit prey response behaviors and will spatially avoid the novel invasive predator. The results suggest that native anurans may have a redundant predator response in both the larval and adult life stages, which could reduce the predatory impact of *X. laevis* but also drive emigration of native amphibians from invaded habitat.

Introduction

Invasive species threaten biodiversity worldwide and reduce or even eliminate populations of native species (Mack et al. 2000; Bellard et al. 2016). Amphibians, in particular, have suffered severe population losses from invasive species owing to disease transmission, competition, habitat alterations, and direct predation (Kats and Ferrer 2003; Bucciarelli et al. 2014). Although many prey taxa have evolved the capacity to detect and respond to the presence of their natural predators to reduce predation risk, naïve native prey species may lack the ability to recognize or effectively respond to introduced predatory species (Sih et al. 2010). A native species may not respond appropriately, particularly if the exotic predator is not closely related to the animal's native predators (Ferrari et al. 2010). Novel invasive predatory species such as introduced mammalian predators in New Zealand (Innes et al. 2010; Goldson et al. 2016) and introduced snakes in Guam (Fritts and Rodda 1998; Wiles et al. 2003) can cause precipitous loss of native species. Similarly, native amphibians in parts of the western United States have declined or disappeared owing to the introduction of predatory bullfrogs and fish species (Knapp and Matthews 2000; Adams and Pearl 2007).

Although studies have explored the responses of anurans to a suite of invasive species to determine if the native species respond to novel predators (e.g. (Gall and Mathis 2010; Nunes et al. 2012; Pease and Wayne 2013), these studies have focused on a single anuran life stage, either the adult or larval stage. But in each life stage the species may be adapted to detect and evade different predators. As a consequence, it is important to include both larval and adult anuran life stages when evaluating the impact of an invasive species because one

life stage may be more vulnerable to predation or less able to respond to the novel predator than the other.

The African clawed frog (*Xenopus laevis*) invasion into southern California (Crayon 2005) offers an opportunity to investigate the responses of native anurans to a novel predatory species through their life cycle. As a generalist predator that preys on its own eggs and larvae (McCoid and Fritts 1980; Measey and Tinsley 1998) *X. laevis* adults could potentially attack and prey upon native amphibian eggs, larvae and adults. Larval *X. laevis,* on the other hand, are not considered a predatory threat to native amphibians because they are obligatory filter feeders (Seale 1982) and were therefore not included in this study. Native to sub-Saharan Africa (Tinsley et al. 1996), the invasion of *X. laevis* into southern California has coincided with declines in native amphibians (Mahrdt and Knefler 1972; McCoid and Fritts 1980); however, it is not clear if *X. laevis* predation was a factor in these declines (Crayon 2005).

Of the dietary studies involving invasive *X. laevis* populations (McCoid and Fritts 1980; Measey 1998; Lobos and Measey 2002; Faraone et al. 2008; Lillo et al. 2011), only one study has shown that *X. laevis* will consume native amphibians (Amaral and Rebelo 2012). Few of these studies, however, reported the presence of native amphibians in the ponds where *X. laevis* were collected (McCoid and Fritts 1980; Measey 1998; Lillo et al. 2011). One study in Italy showed that several amphibian species (*Hyla intermedia*, *Pelophylax esculentis*, *Discoglossus pictus*), but not all species (*Bufo bufo*), stopped reproduction in ponds after *X. laevis* had established (Lillo et al. 2011). This suggests that native anurans that are susceptible to predation either emigrate from invaded waters to avoid predation or are quickly consumed, resulting in local extirpation

Southern California amphibians have co-evolved with a variety of native anuran predators. For example, adult California red-legged frogs (*Rana draytonii*) are known to prey upon Pacific chorus frogs (*Pseudacris regilla*) (Hayes and Tennant 1985) and southern mountain yellow-legged frogs (*Rana muscosa*) are thought to feed on *P. regilla* and *Anaxyrus* (*Bufo*) species (Pope and Matthews 2002). *X. laevis* is comparable in size to these predatory anurans, but belongs to a family (*Pipidae*) that is not native to North America, which leaves a long evolutionary gap for potential prey recognition. Invasive *X. laevis* are also not typical of California frogs because they are nearly fully aquatic, leaving water only occasionally for dispersal (Lobos and Jaksic 2005). Their lack of tongue and suction feeding strategy are more typical of a fish than a native amphibian (Measey 1998). *P. regilla* do respond to fish predators (Pearl et al. 2003) but may not associate novel amphibian cues with a fish-like predatory threat. As a consequence, native amphibians may not recognize or appropriately respond to *X. laevis* in their adult or larval life stages.

This study used Pacific chorus frogs (*Pseudacris regilla*) as a representative of native California species to explore potential predation by and behavioral responses to *X. laevis*. *P. regilla* was chosen because it is highly palatable to most predators, is common throughout southern California, and has declined in areas *X. laevis* has invaded (Mahrdt and Knefler 1972; McCoid and Fritts 1980). Given that both larval and adult *P. regilla* occupy the slow-moving aquatic habitats favored by *X. laevis* (Crayon 2005), it is important to evaluate the potential effect of *X. laevis* on both larval and adult *P. regilla* life stages.

The following experiments were designed to determine (1) whether *X. laevis* prey upon *P. regilla* larvae and adults and (2) whether *P. regilla*, larvae or adults, respond to *X. laevis* with anti-predator behavior. Understanding the predation risks and responses will

serve as a first step towards assessing the impact *X. laevis* may have on native anurans, and the potential for coexistence of native anurans with *X. laevis*.

Materials & Methods:

Experiments were performed over two summer field seasons using animals collected from the wild. The *P. regilla* and *X. laevis* were kept in plastic aquaria (18 cm wide, 17cm tall, 28 cm long), filled with 4L of Nestle® bottled drinking water for adult *X. laevis*, larval *X. laevis*, and larval *P. regilla*, whereas adult *P. regilla* were supplied a water dish, unless otherwise stated. Animals were fed twice per week and tanks were cleaned weekly. Larval *P. regilla* were fed flake fish food and algal pellets; adult *P. regilla* were fed live crickets; and adult *X. laevis* were fed *Xenopus*-specific Nasco® frog brittle.

X. laevis* Predation on Larval *P. regilla

Laboratory experiments were performed to determine if adult *X. laevis* would feed on larval *P. regilla*. The *P. regilla* larvae were raised from egg clutches deposited in the laboratory containers by amplexed adult pairs collected from Atascadero Creek, Santa Barbara County. Nine adult *X. laevis* were collected from a pond in the Hedrick Ranch Nature Area (HRNA), which is adjacent to the Santa Clara River, Ventura County, and kept in laboratory aquaria. One adult *X. laevis* was excluded from the study because it had not been observed feeding in the days leading up to the experiment and may have been sick at capture or particularly distressed from captivity.

The eight remaining *X. laevis* were fasted for five days and measured (snout-to-vent length, SVL) before the predation trials. A *P. regilla* larva (stages 32 to 41) (Gosner 1960)

was haphazardly selected and placed in one of the eight *X. laevis'* tanks. When the *X. laevis* consumed a *P. regilla*, another larva was placed in the tank within five minutes. The *P. regilla* were continuously replaced if consumed. Each trial ended when the *X. laevis* did not consume the *P. regilla* larva within ten minutes of its introduction. We analyzed the relationship between the size of each *X. laevis* and the number of *P. regilla* it consumed using a linear regression.

X. laevis Predation on Adult and Juvenile *P. regilla*

A laboratory experiment was performed to determine if adult *X. laevis* would feed on adult and juvenile *P. regill*a. The eight adult *P. regilla* used in this experiment were collected from Atascadero Creek, Santa Barbara County. The juvenile *P. regilla* were the remaining metamorphosed larvae from the previous *P. regilla* larva predation experiment never exposed to a *X. laevis*. The sixteen *X. laevis* were collected from the isolated pond at the HRNA, eight of which had been used in the previous larval *P. regilla* predation experiment.

The *X. laevis* were fasted for two days before the trials. One *X. laevis* and one adult or juvenile *P. regilla* were measured and placed in an aquarium (18 cm wide, 17cm tall, 28 cm long) filled with 5.5L of bottled water with approximately 4cm of space between the water surface and a fine mesh lid. The *X. laevis* were evaluated based on whether or not the *P. regilla* was consumed within a 24 hour period. Two *X. laevis* that did not consume a *P. regilla* were re-tried with smaller *P. regilla*.

Behavioral Response of Larval *P. regilla* to *X. laevis* and a Native Invertebrate Predator

A laboratory experiment was performed to determine if *P. regilla* larvae would exhibit an anti-predatory behavioral response in the presence of adult *X. laevis* (a non-native predator) and dragonfly nymphs (Aeshnidae; a native predator) as measured by larval activity levels and spatial avoidance. The *P. regilla* larvae were collected from a shallow isolated artificial pool lacking predators located in the HRNA, then kept in an aerated 20 gallon glass aquarium at 24°C. Four of the *X. laevis* were collected from a pond on HRNA and four from an isolated pool on Piru Creek, a tributary of the Santa Clara River, in Ventura County. The four *X. laevis* collected from HRNA were later used in the *P. regilla* predation experiments. Eight dragonfly nymphs were collected from an isolated pool on the UCSB campus and kept in the laboratory in individual aerated plastic containers with 0.5L of bottled water and fed bloodworms twice a week.

Trials were performed in clear plastic aquaria (18 cm wide, 17cm tall, 28 cm long), each divided into two equal chambers by a clear plastic mesh divider (1.5 mm gauge). The tanks' sides were covered in a white translucent screen to reduce shadows. Four liters of fresh bottled water at 25±1.5°C were used in each trial. Tanks were wiped down with 10% bleach and rinsed repeatedly (approximately five times) with DI water between each trial to remove residual animal cues.

A *P. regilla* larva was placed into each of the five treatments: *X. laevis* present (n=22), *X. laevis* scent (n=24), dragonfly present (n=26), dragonfly scent (n=18), or a control (no predator or predator scent; n=25). In the *X. laevis* and dragonfly present treatments, the predator was placed in the trial aquaria for 30 minutes and the water was stirred prior to the introduction of the *P. regilla* larva to the opposing chamber. In the *X. laevis* and dragonfly scent treatments, the predator was placed in the aquarium for 30 minutes, removed, and the water stirred prior to the introduction of the *P. regilla* larva to the opposing chamber. Each

P. regilla larva was staged following Gosner (Gosner 1960) and used only once. The *X. laevis* adults and dragonfly nymphs were used repeatedly in trials.

Trials were conducted between 1100 h and 1600 h and each ran for eleven minutes. The trials were videotaped from 20 cm above the water using a digital camera and later analyzed to evaluate *P. regilla* activity levels and spatial distribution within the aquarium. The first minute of each trial was discarded as an acclimation period for the *P. regilla* larva. In the following ten minutes, we summed the number of seconds each *P. regilla* larva was active and the number of seconds it spent in the half of the aquarium closest to the mesh divider. Larvae were considered active if their tails were in motion. All time measurements were rounded to the nearest second for each bout of activity or move from one half of the aquarium to the other.

Separate ANOVAs and *post hoc* Tukey tests were performed on the number of seconds the *P. regilla* were active and the number of seconds they spent on the half of the tank closest to the mesh divider to compare treatment effects on activity levels and spatial avoidance, respectively.

Behavioral Response of Adult *P. regilla* to *X. laevis*

A field enclosure experiment was performed to determine if adult *P. regilla* would spatially avoid adult *X. laevis*. Thirty new adult *P. regilla* were collected from Isla Vista and Atascadero Creek, Santa Barbara County. Twenty new adult *X. laevis* were collected from a pond on HRNA.

Fifteen enclosures were used in the field experiment (Figure 2.1). Each enclosure consisted of a rectangular 3-dimensional PVC frame (45 cm wide, 22 cm tall, 58 cm long) with small gauge (1cm) plastic mesh on the top and on the four sides. The bottom of the

enclosure was natural dirt with two aquaria (18 cm wide, 17cm deep, 28 cm long) buried flush with the ground and filled with 7L of well water, acting as separate water bodies. The two aquaria were arranged in the enclosure to create two halves, each with a water source and equal areas of substrate.

Fifteen enclosures, five control without *X. laevis* and ten treatment enclosures with *X. laevis* were placed, single file, in an open area in HRNA, with every third enclosure a control. One adult *P. regilla* was included in each treatment and control enclosure. In each treatment enclosure, one *X. laevis* was placed in a mesh cube (20 cm by 15 cm by 15 cm) in the aquarium on one side of the enclosure, and an empty mesh cube was placed in the aquarium on the other side (alternating sides in each treatment enclosure). In this way, the *X. laevis* was confined to the mesh cube in one of the aquaria in the treatment enclosures but the *P. regilla* had free movement within the entire enclosure and could use either of the aquaria as a water source.

The experiment was run twice, each for six days, first with all male *P. regilla* and the second with all female *P. regilla*. Different *X. laevis* were used in each run of the experiment. At the beginning of the experiment, the *P. regilla* was placed in the center of each enclosure 30 minutes after a *X. laevis* had been placed in the mesh aquarium cube in each treatment enclosure. The position of each *P. regilla* was recorded three times per 24 hours, once at dusk, once in the middle of the night at 0100 h and once at pre-dawn. The experiments began at 0100 h on the first day and ended with a pre-dawn observation on the sixth day, for a total of 17 observation periods. Each enclosure was checked in succession and no more than 3 minutes was spent locating the *P. regilla* in each enclosure.

The data were statistically analyzed with sign tests based on which side of the enclosure each individual *P. regilla* preferred. For each *P. regilla*, we summed the number

of times it was observed on each side of the enclosure: the non-*X. laevis* or *X. laevis* side of the enclosure for the treatments; or the East or West side of the enclosure for the controls. We assigned a preference for one side or the other based on which side of the enclosure the *P. regilla* was observed on more often. Similarly, an additional sign test was performed to determine a preferential use of either of the water sources within the control and treatment enclosures using only the observation points when *P. regilla* were observed in the water.

Results

X. laevis Predation on Larval *P. regilla*

There was a significant positive correlation between the size of the *X. laevis* and the number of *P. regilla* larvae consumed (linear regression, $R^2 = 0.83$, $p < 0.05$) (Figure 2.2). Seven out of the eight adult *X. laevis* consumed at least one *P. regilla* larva during the trial. One *X. laevis* individual consumed 25 *P. regilla* larvae in the time of the experiment, approximately 3.5 hours.

X. laevis Predation on Adult and Juvenile *P. regilla*

The *X. laevis* consumed 15 of the 18 adult and juvenile *P. regilla* within the 24-hour feeding trials (Figure 2.3). All juvenile *P. regilla* (SVL between 14 and 24 mm) were consumed by *X. laevis* (SVL 33-102mm). The three largest adult *P. regilla* (SVL 34-35mm) were not consumed.

Behavioral Response of Larval *P. regilla* to *X. laevis* and a Native Invertebrate Predator

There was a significant effect of predator treatment on *P. regilla* larvae activity levels (ANOVA: $F_{1,4}$=4.25, p<0.01). However, no activity levels in any of the treatment were found to be significantly different from the control. The *post hoc* analysis found significant differences only between the *X. laevis* scent treatment and both the dragonfly nymph present and dragonfly nymph scent treatments (Figure 2.4) (Tukey HSD, p<0.05). For both *X. laevis* and dragonflies there is a trend for increased activity in the presence of the predator compared to the presence of only the scent of the predator; however, these differences are not statistically significant for either predator species.

There was a significant effect of predator treatment on spatial distribution of larval *P. regilla* within the aquaria (i.e. on the time spent in the half of the aquarium closest to the mesh divider vs. the back half of the aquarium; ANOVA: $F_{1,4}$=3.57, p<0.001). The *post hoc* analysis found significant differences between the control and the *X. laevis*-present treatment, with the *P. regilla* spending significantly less time in the half of that aquarium that was closer to the predator (Figure 2.5; Tukey HSD, p<0.05).

Behavioral Response of Adult *P. regilla* to *X. laevis*

Adult *P. regilla* in the absence of *X. laevis*, displayed no preference for either side of the enclosures (binomial test: n=10, p>0.05) (blue dots in Figure 2.6a). Five *P. regilla* in the control enclosure were observed more often on the East side and five were observed more often on the West side of the enclosure. All control *P. regilla* were observed at every survey period.

P. regilla in the treatment enclosures displayed a significant preference for the side of the enclosure without the *X. laevis* (binomial test: n=20, p< 0.001) (red triangles Figure 2.6a). All 20 *P. regilla* in the treatment enclosures were observed more often on the non-*X.*

laevis side of the enclosure than the *X. laevis* side. The *P. regilla* moved throughout the enclosure, rarely observed in the same location for two sequential time points in the treatment or control enclosures. On two occasions, a *P. regilla* in a treatment enclosure could not be located within the enclosure and those individual *P. regilla* have 16 rather than 17 observations.

The *P. regilla* also displayed a significant preference for the non-*X. laevis* water source in the treatment enclosures (binomial test: $n=16$, $p<0.001$) and showed no preference for the East or West water sources in the control enclosures (binomial test: $n=8$, $p>0.05$) (Figure 2.6b). *P. regilla* that were not observed in water or were observed an equal number of times in both water sources were not included in this statistical analysis. There does not appear to be a pattern for when the *P. regilla* were observed in the *X. laevis* water source; some individuals were observed in the *X. laevis* water source in the beginning, middle, and end of the experiment. Only one female *P. regilla* was observed in a *X. laevis* water source, on one occasion, whereas five males were observed a total of seven times in *X. laevis* water source.

Discussion

Our predation experiments suggest that invasive *X. laevis* will prey upon larval and adult *P. regilla*. This is unsurprising given that larval *P. regilla* are a similar size to *X. laevis* larvae, which are cannibalized (Tinsley and McCoid 1996). Of greater concern for native amphibian populations is the ability of *X. laevis* to consume the adult *P. regilla*, which may have more profound population consequences. The loss of later life stages and older reproductive individuals may cause greater declines in populations than the loss of eggs or

young juveniles (Doak et al. 1994; Vonesh and la Cruz 2002). Larval anurans suffer high mortality rates, some over 95% (Herreid and Kinney 1966), which means the few that survive to reproductive age become increasingly important to create the next generation. Only the largest adult *P. regilla* (34-35mm SVL) avoided predation when matched up with small *X. laevis* (<70mm SVL), presumably owing to gape limitation of the smaller adult *X. laevis*. However, gape limitation is unlikely to limit the larger *X. laevis* that reach >100mm (SVL).

Although larval and adult *P. regilla* life stages were consumed by *X. laevis* in the laboratory, this does not necessarily imply that *X. laevis* predation will reduce or extirpate *P. regilla* populations in the field, although there is some evidence of this occurring (Mahrdt and Knefler 1972; McCoid and Fritts 1980). Amphibians can reduce their detection and capture by predators through a variety of anti-predator behaviors. Laval amphibians exhibit spatial avoidance, increased refuge use, as well as changes in morphology, timing of metamorphosis, and activity level (Skelly and Werner 1990; Pearl et al. 2003; Hossie et al. 2010). Similarly adult anurans are selective in where they lay their eggs and have been shown to avoid ovipositing in waters with predators (Rieger et al. 2004). These anti-predator responses should be expressed only in the presence of a threat because the behaviors or changes in morphology tend to be costly. For example, reduced larval activity reduces the amount of time larvae spend foraging and thereby adversely affects their later size, survivorship, growth, and development (Skelly 1992).

In this study, *P. regilla* displayed a spatial avoidance response to predator presence but did not display changes in activity levels. It is not surprising that the larvae displayed only one of the two response behaviors tested. Anuran larvae have been shown to have specific responses for different predators (Relyea 2001) and often the presence of more

predator cues results in a stronger prey response than one predator cue alone (Hettyey et al. 2012). The *X. laevis* scent cue may trigger a response that was not measured in this study, such as changes in tail morphology or accelerated time to metamorphosis.

The larvae's spatial avoidance response likely results from general predator cues that happen to fit *X. laevis* rather than cues specific only to *X. laevis*. The changes in water movement or an approaching dark shape could have caused the *P. regilla* to respond to the presence of *X. laevis*. There were multiple occasions when the predator appeared to have observed the *P. regilla* larva on the other side of the mesh and lunged towards it, which often caused the *P. regilla* to quickly swim away. This predator behavior could have influenced the spatial distribution of the *P. regilla* larvae because it only occurred near the mesh divider.

Predator motion could also explain the differences seen in the activity level between the predator present and predator scent-only treatments. For both *X. laevis* and dragonflies as predators, there was a tendency for *P. regilla* larvae to be more active in the presence of the predators than in the presence of just the predator scent. The motion of the predator appeared to be necessary for the *P. regilla* to initiate a behavioral response. The *P. regilla* did not appear to respond to the visual outline of the *X. laevis* as a potential threat; the larvae would often approach a motionless *X. laevis* or rest immediately next to the *X. laevis* on the opposite side of the mesh divider.

Xenopus laevis may be too far removed evolutionarily from *P. regilla's* natural predators, for *P. regilla* to recognize its specific scent or other *X. laevis*-specific cues as a potential threat. Although *P. regilla* did evolve with native anuran predators (Hayes and Tennant 1985; Pope and Matthews 2002), more distantly related animals are thought to have more dissimilar scent cues that limit an amphibian's ability to recognize novel predators (Ferrari et al. 2010). This has been shown with other amphibian species when exposed to

novel predatory fishes with varying relatedness to their native predators (Gall and Mathis 2010). The Pipidae family, which includes *X. laevis*, is native to Africa and South America and *Xenopus* is endemic to sub-Saharan Africa (Tinsley and Kobel 1996). The olfactory scent cues may not be recognized by *P. regilla* larvae, which could force the larvae to rely upon more general predator cues that matched a native predator in some way. Native predatory anurans (*Rana draytonii* or *Rana muscosa*) were not included in this study due to their sensitive population statuses, and no other study was found to explore the behavioral responses of *P. regilla* to native anuran predatory species.

It is unclear why *P. regilla* larvae did not respond significantly to the native dragonfly nymphs, a native predator. Other anuran species have displayed reduced activity levels in the presence of native dragonfly nymphs (Nunes et al. 2012), and *P. regilla* larvae have been shown to spatially respond to dragonfly nymphs (Hammond et al. 2007). In our study, the data suggest that *P. regilla* larvae may increase their activity levels and spatially avoid dragonfly nymphs when in their presence, perhaps in effort to leave the area. However, the increased activity did not significantly differ from the control and may be a product of the attack motions of the nymphs eliciting flight reactions from the larvae.

Larval *P. regilla* might have exhibited stronger responses to the presence of the dragonfly and *X. laevis* predators were the scent of a consumed conspecific present. Anuran larvae sometimes respond to novel predators when a cue from consumed conspecifics is present, either through the diet of the predator or the presence of the larvae's broken skin (Marquis et al. 2004; Mandrillon and Saglio 2005). This response could compensate for the lack of a species-specific predator response, allowing native larvae to avoid a range of predators without recognizing them individually. Neither predator in this experiment was fed

anurans as part of their diet in order to eliminate this as a potential factor, but it may be key to stimulating a behavioral response.

Adult *P. regilla* displayed a significant spatial avoidance behavior when exposed to *X. laevis*. The adult behavioral response may be more important than the larval response because the adults can reduce the larvae's exposure to the *X. laevis* by discriminating between invaded and non-invaded sites when selecting breeding sites. If the parents avoid depositing eggs in areas with aquatic predators, then the larvae have less need for an innate anti-predator response because they are not frequently exposed to those predators. Other naïve anuran species in Europe have been shown to stop reproduction in ponds after establishment of invasive *X. laevis* (Lillo et al. 2011). The data from the field enclosure experiment suggests that *P. regilla* avoid *X. laevis* invaded water sources when possible, because *P. regilla* were rarely observed in the water with a penned *X. laevis*. This experiment was performed during the breeding season of *P. regilla*, which suggests that *P. regilla* may avoid *X. laevis* areas when ovipositing, if alternative sites were available.

Xenopus laevis displays a clear ability to consume both larval and adult stages of native amphibians and may indirectly cause native amphibian emigration from local habitats through spatial predator avoidance. Native California anurans are absent from many areas that *X. laevis* has invaded but have never been found in the stomach contents of *X. laevis* (Crayon 2005), suggesting either quick consumption to extirpation or emigration of native anurans from those areas. These potential impacts may warrant *X. laevis* management to limit their current populations and prevent further invasions. Active management aimed at preventing their introduction is ideal because they are difficult to eradicate once established (Crayon 2005), although *X. laevis* populations have been shown to decline or go extinct on their own due to extreme cold or dry weather conditions (Rebelo 2010; Tinsley et al. 2015).

The recent drought in southern California may have eliminated some populations as semi-permanent water sources dried. Further study will be necessary to determine if co-existence between native amphibians and *X. laevis* occurs in the wild.

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Tables & Figures

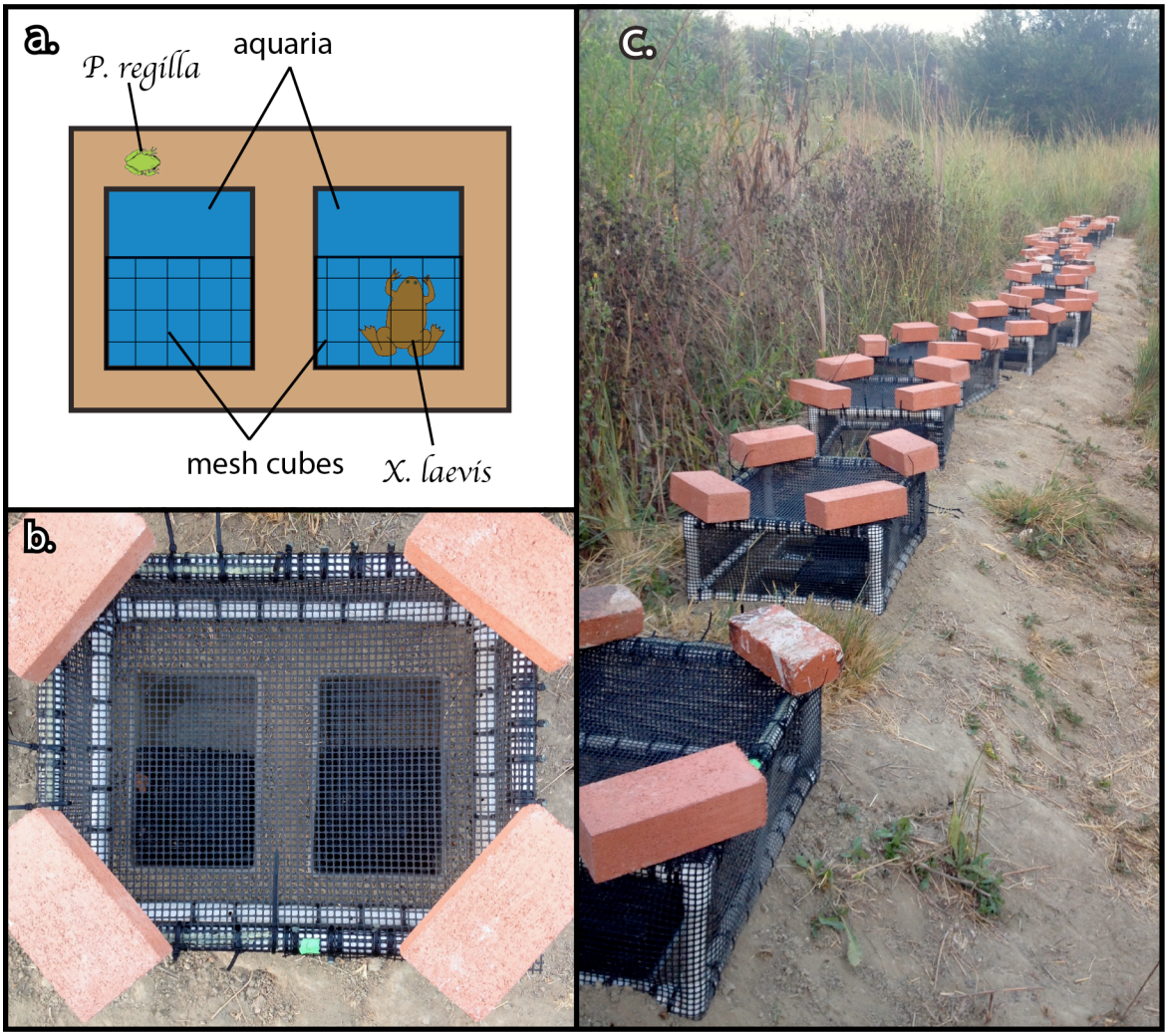

Figure 2.1. Field enclosure setup for adult *P. regilla* behavioral experiment
A diagram showing an overhead view of the inside of a treatment enclosure with a *X. laevis* penned in the right aquarium and the *P. regilla* on the opposite (non-*X. laevis*) side of the enclosure (a.); a top view of a treatment enclosure (b.); the 15 enclosures set up in the field (c.). Bricks were used to hold the enclosures in place.

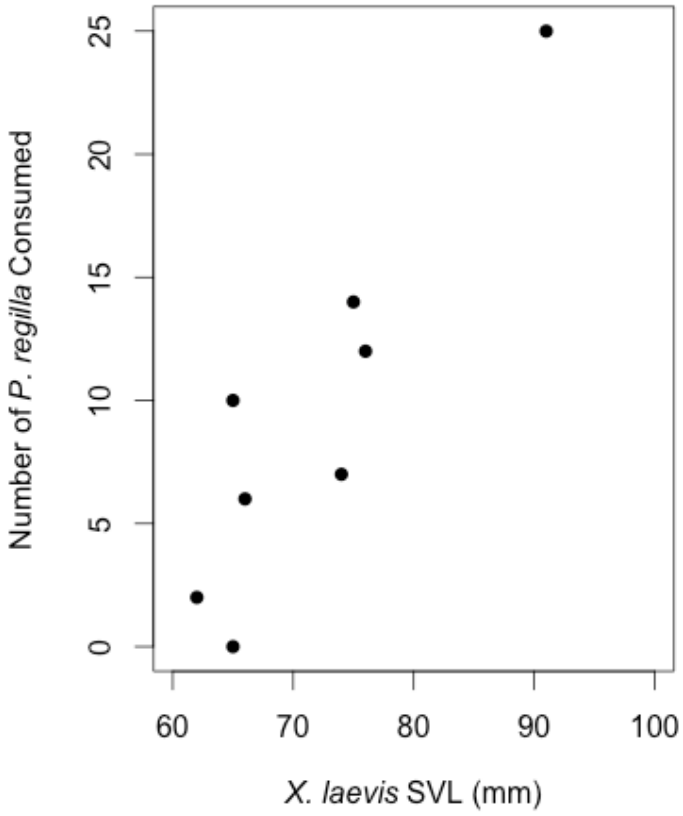

Figure 2.2. Number of larval *P. regilla* consumed by *X. laevis*
The number of *P. regilla* larvae (Gosner stage 32-41) consumed by each *X. laevis* in the approximately 3.5 hour predation trial (SVL=snout to vent length).

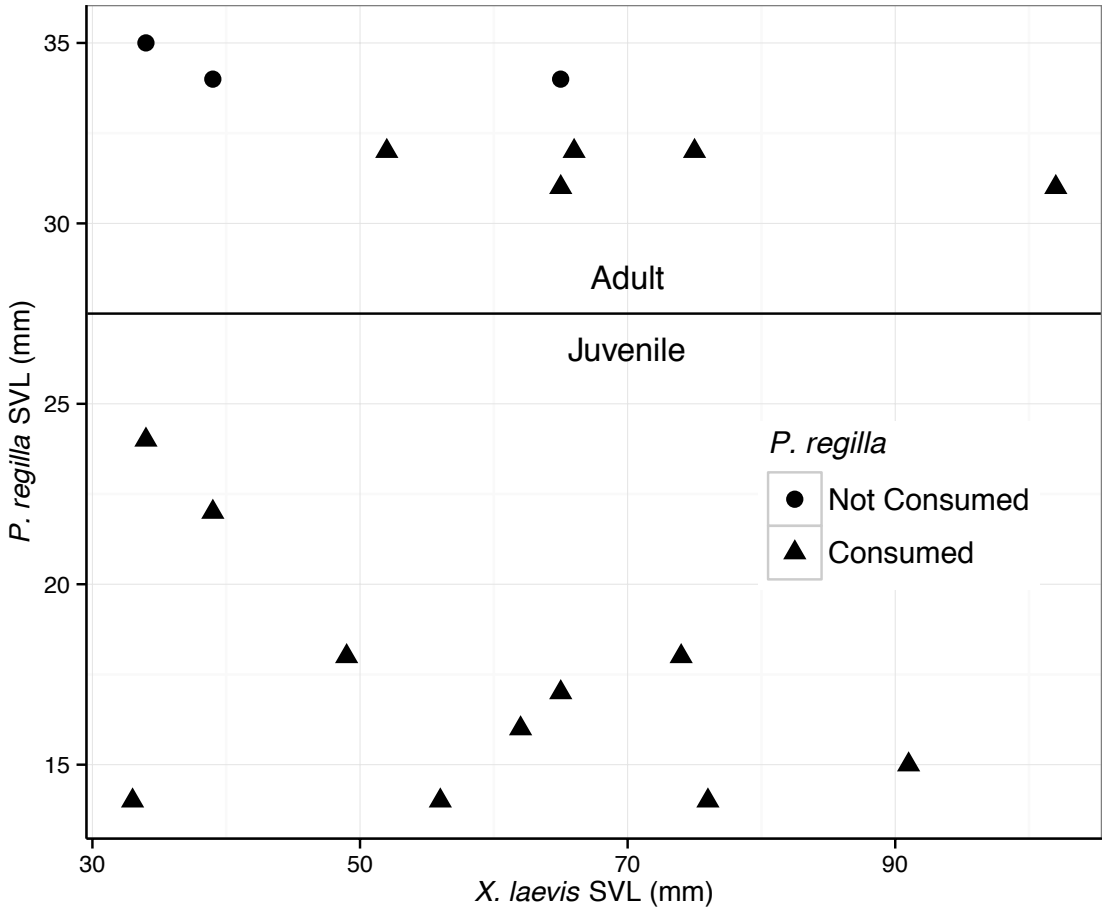

Figure 2.3. Size of adult and juvenile *P. regilla* consumed by *X. laevis*
Consumption of adult and juvenile *P. regilla* by *X. laevis* of various sizes in a 24-hour predation trial (SVL=snout to vent length).

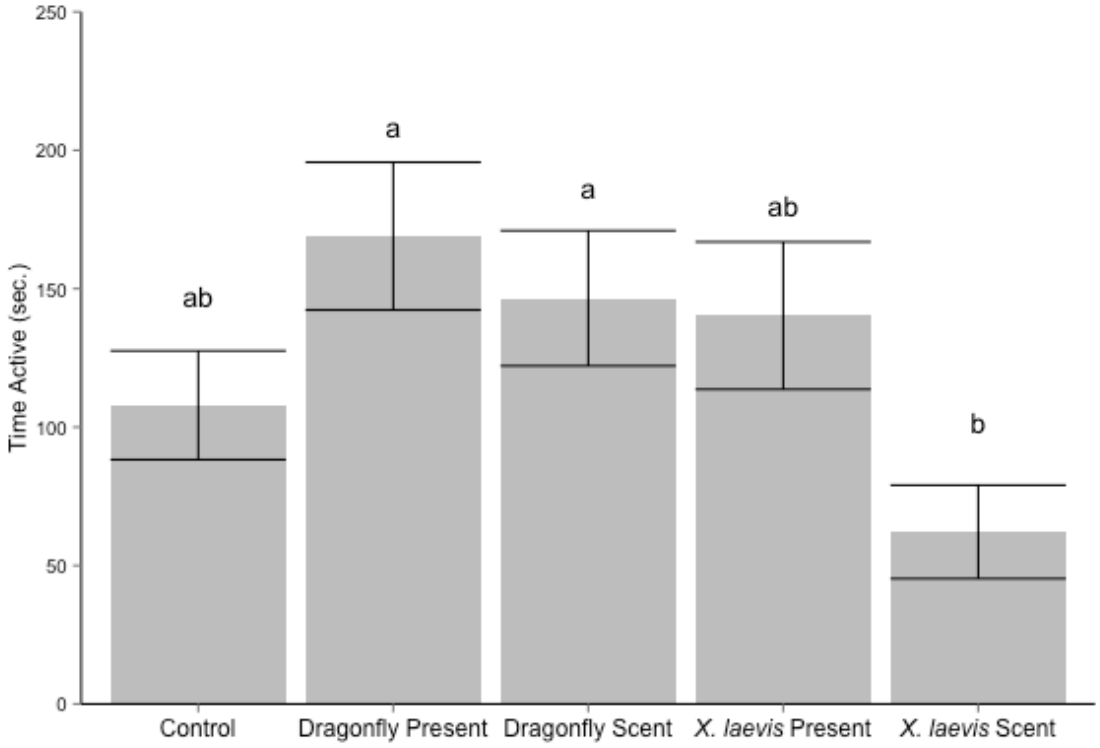

Figure 2.4. Larval *P. regilla* activity levels
Activity levels (mean ± SE) of the *P. regilla* larvae when exposed to the scent cues or presence of a dragonfly nymph or adult *X. laevis*. Letters above SE bars distinguishing statistical differences between treatments and/or the control (Tukey pairwise comparison, $p<0.05$).

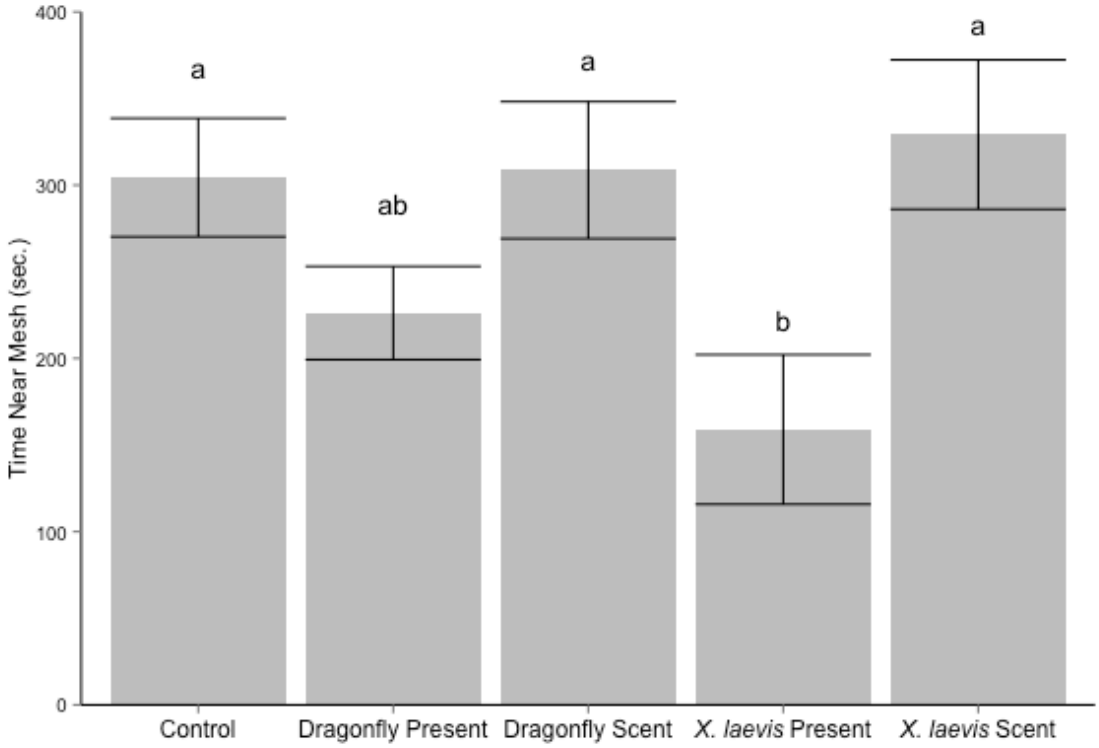

Figure 2.5. Larval *P. regilla* spatial distribution
Spatial distribution of the *P. regilla* larvae when exposed to the scent cues or presence of a dragonfly nymph or adult *X. laevis*; number of seconds (mean ± SE) the *P. regilla* larvae spent on the half of the aquarium closest to the center mesh divider (near the predator). Letters above the SE bars distinguishing statistical differences between treatments and/or the control (Tukey pairwise comparison, $p<0.05$).

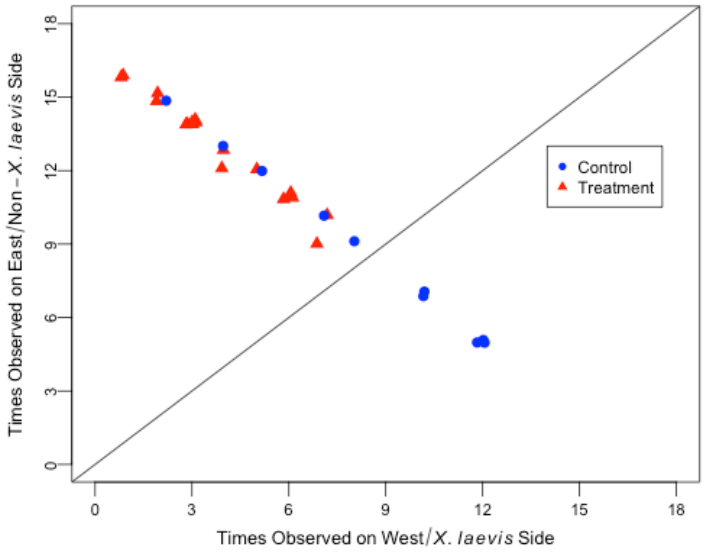

Figure 2.6a.

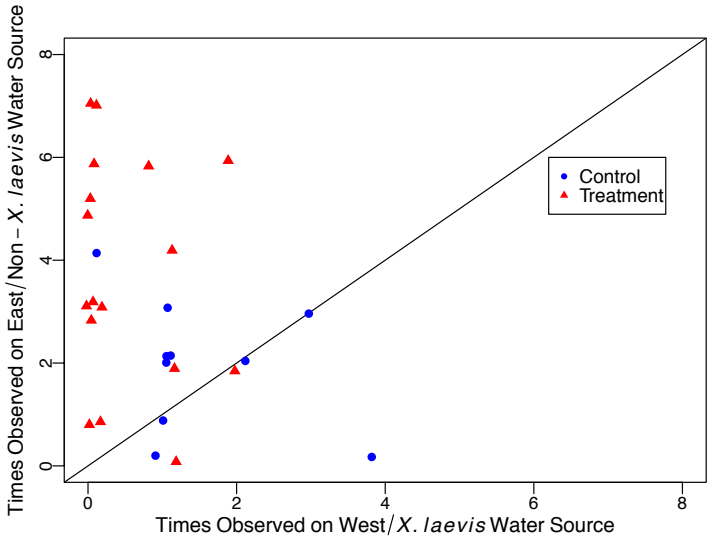

Figure 2.6b.
Adult *P. regilla* spatial distribution
The number of times each adult *P. regilla* was observed on either side of the enclosure (2.6a) or in the water sources (2.6b). *P. regilla* in control enclosures were observed on either the East or West side and *P. regilla* in treatment enclosures were observed on either the non-*X. laevis* or the *X. laevis* side. Points were offset slightly to distinguish individuals with identical observation counts. The diagonal line represents an equal number of observations on either side of the enclosure or water source.

Chapter 3

The Use of Environmental DNA for Detection of Cryptic African Clawed Frogs and Co-occurrence with Native Amphibians

Abstract

The invasion of African clawed frogs (*Xenopus laevis*) has followed noted declines of native amphibian species in southern California. Native amphibians are known to spatially avoid *X. laevis* and may leave invaded areas, effectively reducing the habitat available to them. This study explores the distribution of *X. laevis* and its co-occurrence with native amphibian species to evaluate potential exclusion of native amphibians in areas with *X. laevis*. Environmental DNA survey methods were used to detect amphibian presence in sites throughout southern California using both a *Xenopus*-specific primer, to detect *X. laevis*, and amphibians-specific primer, to detect all amphibian species. Native amphibians were found to co-occur with *X. laevis* at multiple sites, suggesting that native amphibians remain in streams following *X. laevis* invasion. The eDNA survey results from the *Xenopus*-specific primer detected *X. laevis* at sites throughout southern California, including two sites where *X. laevis* have not previously been documents; however, the survey failed to detect *X. laevis* at six sites where they have historically been present. While there may have been inhibitors in the eDNA samples that may have limited the detection of amphibians at some sites, the results suggest that native amphibians are able to co-exist with *X. laevis* in streams.

Introduction

Environmental DNA (eDNA) is a valuable tool to detect a species presence and could replace more traditional survey methods (Thomsen and Willerslev 2015). By sampling the DNA animals leave behind in the environment (tissue, blood, mucus, etc.), eDNA methods can detect the presence of a targeted taxon's unique genetic sequence without the labor intensive methods that rely upon visual or acoustic recognition of a species. Aquatic species lend themselves to this method of detection because animals shed tissue directly into the water, which can subsequently be sampled and filtered to capture cells and even organelles (Turner et al. 2014). By amplifying small amount of genetic tissue, it is possible to detect rare, sensitive, or cryptic species in complex aquatic habitats without disturbing target or non-target species, damaging the habitat, or devoting extensive work hours in the field (Beja-Pereira et al. 2009).

Molecular methods to detect species have been used previously to detect ancient organisms in soil or ice samples or to detect microorganisms presence in work related to water quality (Thomsen and Willerslev 2015). More recently these molecular techniques are being adopted to detect aquatic macroorganisms that are currently in the environment but difficult to observe (Ficetola et al. 2008). DNA slowly degrades in water but persists for days to weeks, restricting detection from water samples to species currently or recently in the water (Dejean et al. 2011; Thomsen et al. 2012a). Technological advances have reduced the costs of these molecular techniques, allowing wider application in many aquatic systems (Taberlet et al. 2012).

This study takes advantage of eDNA techniques to survey the distribution of amphibians in southern California an order to evaluate whether native amphibians co-exist with invasive African clawed frogs (*Xenopus laevis*). Initially shipped globally for medical

and then scientific work (Crayon 2005), *X. laevis* has established invasive populations in Chile (Lobos and Measey 2002), western Europe (Italy, Spain, Wales) (Measey 1998; Fouquet and Measey 2006; Faraone et al. 2008), Japan (Kobyashi et al. 2005), Mexico (Peralta-García et al. 2015), and the United States (Tinsley and McCoid 1996). Invasive populations of *X. laevis* are considered harmful to native ecosystem and possession of the species is restricted in California (14 U.S.C. §671). They have been found to prey upon native amphibians (Amaral and Rebelo 2012; Chapter 2) and endangered fish (Lafferty and Page 1997) and harbor an amphibian pathogen that has driven amphibian population declines globally (Weldon et al. 2004; Fisher and Garner 2007; Soto-Azat et al. 2010).

The invasion of *X. laevis* has been followed by noted decline of native amphibians in some areas (Mahrdt and Knefler 1972; Lillo et al. 2011) and behavioral experiments suggest that amphibians native to California may spatially avoid *X. laevis* (Chapter 2). To date, only Lillo et al. (2012) have explored the effect of *X. laevis* invasion on native amphibian diversity. It found a significant decline in native Sicilian amphibians as *X. laevis* became established in ponds. In southern California, *X. laevis* have established populations in watersheds with threatened and endangered amphibians species such as the California red-legged frog (*Rana draytonii*), arroyo toad (*Anaxyrus californicus*), and California treefrog (*Pseudacris cadaverina*) (Tinsley and McCoid 1996), increasing the urgency to understand whether *X. laevis* are causing declines of native amphibians.

Environmental DNA is the ideal technique to detect the presence of amphibian species and can be used to address the questions of co-occurrence on a scaled that would not be possible using traditional survey techniques. The time necessary to survey for the amphibians species using traditional methods such as seines, traps, etc., on repeated site visits (Graeter et al. 2013), make large-scale surveys for all aquatic amphibian species

challenging. In addition, many of the locations where *X. laevis* have invaded are coastal rivers and streams that harbor sensitive species, such as steelhead, and therefore have restrictions on animal capture methods that make it difficult to effectively survey. However, by collecting only water samples from the field, eDNA survey methods avoid these conflicts and reduce the amount of time required to survey large areas.

Environmental DNA techniques can be used to detect a single target species or a broader taxonomic group. In species-specific eDNA detection, a primer pair and an additional probe are designed to detect a short segment of only the targeted specie's DNA among all the other DNA sequences present in an environmental sample. The pair of primers attaches to the targeted species' DNA and through polymerase chain reaction (PCR) methods, amplifies (creates copies) of the DNA segment between the two primers. The probe sequence is designed to match a section within the short DNA segment and fluoresces when successfully attached, allowing detection and quantification in quantitative PCR (qPCR). The amplified DNA can also be detected using traditional PCR with DNA detection using gel electrophoresis. This approach does not use a probe and is not as sensitive to low concentrations of DNA as qPCR (Darling and Mahon 2011). Many studies have successfully used both eDNA methods to detect targeted fish (Jerde et al. 2011; Takahara et al. 2013; Wilcox et al. 2013), amphibians (Ficetola et al. 2008; Goldberg et al. 2011; Olson et al. 2013; Pilliod et al. 2013; Fukumoto et al. 2015; Biggs et al. 2015), or invertebrates (Goldberg et al. 2013; Deiner and Altermatt 2014; Tréguier et al. 2014; Egan et al. 2015) from water samples.

Alternatively, multiple species can be detected using a general primer pair that target a larger taxonomic group (Thomsen et al. 2012a). The amplified DNA segments are then read with next-generation sequencing (NGS) methods (also referred to as metabarcoding or

high-throughput sequencing) and analyzed to distinguish different, but related species in the sample. The primers may include degenerate base pairs to create ambiguous sequences, which expand the diversity of genetic sequences that can be targeted (Minamoto et al. 2011). A number of studies have successfully utilized general primers to detect species diversity in eDNA samples from fishes (Thomsen et al. 2012b; Evans et al. 2015; Miya et al. 2015; Valentini et al. 2016; Olds et al. 2016; Keskin et al. 2016), invertebrates (Deiner et al. 2016) , and amphibians (Evans et al. 2015; Valentini et al. 2016) .

To detect the presence of amphibian species in southern California and evaluate co-occurrence between *X. laevis* and native amphibians, we used eDNA survey methods with a general amphibian primer designed to detect any species in the suborder Batrachia (Anurans and Urodela), created by Valentini et al. (2016). In addition, a species-specific primer, created by Secondi et al. (2016) was used to confirm the detection of *X. laevis* in the eDNA samples and further monitor its range. Both the amphibian-specific and *Xenopus*-specific primers have been used successfully in Europe but have not been tested in other regions. While all primers should be developed in a way that they can be used in other regions, and previous designed primers have been used in different areas with success (Jane et al. 2014; Biggs et al. 2015; Miralles et al. 2016), it is important to validate them in new study areas (MacDonald and Sarre 2016).

In this study, water samples were collected from sites throughout southern California and use the amphibian-specific and *Xenopus*-specific primers to determine the presence of amphibian species. Application of the amphibian-specific and *Xenopus*-specific primers was expected to transfer well to southern California and effectively detect the amphibian species and *X. laevis*, respectively. The survey areas included sites with range of amphibian community assemblages to effectively test the primers' ability to detect different species and

observe patterns of amphibian assemblages related to the presence of *X. laevis*. Sample sites covered coastal areas from the Santa Ynez River estuary to the Tijuana River. These data were collected to help determine the distribution of *X. laevis* and evaluate if native amphibians co-exist with *X. laevis*.

Material & Methods

Primer Specificity Tests

Amphibian-specific primers:

The amphibian-specific primer was tested for specificity to amphibian DNA by Valentini et al. (2016) using sequences available in the European Nucleotide Archive (EMBL-Bank) genetic database and tested with DNA extracts from European amphibians. For this study, to confirm that the amphibian-primer detects California amphibian species, tissue extract from amphibians likely to be present in the eDNA samples were tested. Tissue samples from between one and ten individuals from the following species were included in the analysis: *X. laevis*, Pacific chorus frog (*Pseudacris regilla*), California treefrog (*P. cadaverina*), western toad (*Anaxyrus boreas*), arroyo toad (*A. californicus*), American bullfrog (*Lithobates catesbeianus*), California red-legged frog (*R. draytonii*), mountain yellow-legged frog (*R. muscosa*), California newt (*Taricha torosa*) and mouse. All the tissue samples were from individuals collected in southern California with the exception of one mouse tissue sample that was from a laboratory-bred mouse.

The tissue samples were extracted using the Qiagen DNeasy Blood and Tissue Extraction Kit following their standard protocol. The specimen dissection and extractions were performed in the same room used later for portions of the water sample extraction but

surfaces were wiped down with bleach and ethanol repeatedly before water samples entered the area to prevent contamination of eDNA samples.

The tissue samples were assayed by traditional PCR with the primers described by Valentini et al. (2016) to detect amphibian DNA. Twenty-five µL of PCR reaction mixture consisting of 0.6µL of each forward and reverse primer (10µM), 10µL of human DNA blocking primer, 0.12µL of AmpliTaq Gold (Life Technologies), 2.5µL PCR buffer (Life Technologies), 1.2µL dNTP, 3.0µL magnesium chloride, and 0.6µL BSA. PCR was performed on a BioRad PCR thermocycler using the cycle protocol from Valentini et al. (2016). Amplicons were gel-electrophoresed and visualized using a Kodak Gel Logic 200.

A reference database was constructed from the sequences of the amphibian tissue extracts to ensure that the amphibian species likely to be found in the water samples had a unique DNA segment to distinguish each species in the NGS results. The PCR products of each species' tissue extract were purified and sequenced using Sanger methods in the reverse and forward direction (Eton Biosciences). The sequences were analyzed and consensus sequences were created for each species. The consensus sequences for each species were queried in the National Center for Biotechnology Information (NCBI) sequence database to confirm each species' unique DNA sequence.

Xenopus*-specific primers:*

The *Xenopus*-specific primer was tested for specificity by Secondi et al. (2016) using sequences available in the European Nucleotide Archive (EMBL-Bank) genetic database and SPYGEN genetic database, which included additional sequences of amphibians native to Europe. The primers matched *Xenopus* and *Tragulus* genera. All *Xenopus* are native to sub-Saharan Africa (Tinsley et al. 1996) and *Tragulus*, as small ungulate, is only found in

specific regions of Southeast Asia and China (Nowak 1991). Since *Tragulus* are not found in southern California and *X. laevis* is the only species in the *Xenopus* genus known to have invasive populations in California (Crayon 2005), these primers are likely to successfully detect only *X. laevis* in southern California.

For this study, the specificity of the *Xenopus*-specific primer to *X. laevis* was confirmed through standard PCR with gel electrophoresis and quantitative PCR (qPCR) using the same amphibian and mouse tissue samples used to test the amphibian-specific primer. Twenty-five µL of PCR reaction mixture consisting of 1x TaqMan Environmental Master Mix, (Life Technologies), 400 nM of forward and reverse primers, and approximately 50 ng of template DNA. PCR was performed on a BioRad PCR thermocycler using the cycle protocol from Secondi et al. (2016). Amplicons were gel-electrophoresed and visualized using a Kodak Gel Logic 200.

Tissue samples were also assayed by quantitative PCR (Applied Biosystems StepOnePlus) in a 25µL mixture containing 1 X TaqMan Environmental Master Mix, 400 nM of each primer, 250 nM of probe, and approximately 50 ng of DNA template. A serial dilution of *X. laevis* DNA extract and *X. laevis*-based gBlock was included in the qPCR assay to establish limits of detection and set standards. Quantitative standards were created from a synthetic gBlock DNA fragment (Integrated DNA Technologies) based on *X. laevis* sequences downloaded from NCBI with added adaptors for length. The gBlock sequence and other primer sequences used in this study can be found in Appendix 3.1. A serial dilution of the synthetic DNA with a concentration range from 2,800 copies to 3 copies per reaction was tested to determine the threshold of detection. Likewise, a concentration range from 10ng to 8.0^{-5}ng of *X. laevis* DNA was tested by qPCR to establish detection limits. All

work involving the synthetic DNA fragment and PCR products was performed in a designated post-PCR area.

eDNA sample collection, filtration, & extraction

Forty sample locations in coastal southern California from the Santa Ynez estuary to the Tijuana River were chosen to include areas with a range of amphibian species, including sites with and without *X. laevis* (Figure 3.1). Each site was evaluated for the likelihood of *X. laevis* presence using data available from USGS, museum collections, and personal communications with biologists familiar with the areas. Sites were placed into four categories of *X. laevis* likelihood of presence: "present" for sites where *X. laevis* were observed at the time of water sample collection; "likely" for sites where *X. laevis* have previously been collected or sites that are tributaries streams or creeks to these sites; "unknown" for sites where *X. laevis* have not been observed but that are either in highly urban areas close to "present" locations or little amphibian survey work has been conducted; "unlikely" for sites that are geographically removed from known records of *X. laevis* invaded areas, are removed from urban areas, and amphibian or other riparian surveys have not found *X. laevis*. Water samples were collected between April and July of 2016. A detailed list of sample locations can be found in Appendix 3.2.

All bottles, jugs, and caps used in collecting water samples were sterilized with 10% bleach and rinsed thoroughly with tap water between site visits. Bottles, jugs, and their caps were rinsed once using water from the sample site and the poured away from the water source. Water samples were collected from the running portion of streams and creeks or around the perimeter of ponds using a Nalgene container and poured into 10-liter jugs. The

individual collecting the water sample wore clean gloves at each location and did not enter the water. Water samples were kept on ice and transported to the laboratory for filtration.

Three samples and one negative control were filtered for each site within 36 hours of collection using a 1.2 µm pore size (47mm diameter) glass fiber filter (VWR International). Two liters of water was processed through each filter using a tabletop vacuum pump unless the filter clogged with debris before the desired volume was processed. In this case, water was filtered until the filter clogged and as a result, less water was filtered at some sites. The negative control, 500 mL of purified bottled drinking water, was filtered last. The filters were removed from the funnels and stored in individual 2mL vials with enough Longmire solution (Longmire et al. 1997; Renshaw et al. 2014) (~800µL) to saturate them. Samples were stored at -4°C until extraction.

Additional negative controls were taken to further detect potential cross-contamination. Ocean water samples were collected from the UCSB campus, which pulls subsurface ocean water from 1/3 mile off the coast and filters the water through a 2µm pore size filter. Negative controls from the sample collection containers (Nalgene bottles, jugs, and caps) were also taken. Three sets of 500mL of bottles water were processed through the collection containers in triplicate using the same protocol as the field water samples.

A preliminary trial was performed to compare two filter extraction protocols to determine which yielded more DNA, the commercially available Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen), or the phenol-chloroform isoamyl alcohol (PCI) extraction method. Both methods have been used in previous eDNA research (Deiner et al. 2015) but a comparison has not been performed with the 1.2 µm pore size pore size glass fiber filters. Both extraction methods were tried on half and whole filters. A full description of the trial

and results can be found in Appendix 3.3. The PCI extraction method was found to yield higher DNA concentrations so that method was used to process all the samples.

Filters were processed using a phenol-chloroform isoamyl alcohol (PCI) DNA extraction protocol modified from Deiner et al. (2015). A complete description of the PCI protocol can be found in Appendix 3.4. The entire glass fiber filter was used in the DNA extraction to maximize the amount of DNA obtained. The extractions were performed in areas dedicated to pre-PCR work. Forty µL of proteinase K was added to each vial and incubated overnight at 56°C in a rotating rack. Extract negative controls were included alongside batches of eDNA samples. Each samples was extracted eluted into 100 µL of 0.25X TE, and stored at -4°C until further analysis.

eDNA Sample Analysis

Amphibian-Specific Detection with Next-Generation Sequencing

A subset of the eDNA sites and negative controls were selected haphazardly and analyzed with NGS to detect the amphibian species present in the samples using the amphibian-specific primer. The samples were prepared for sequencing by modifying the 16S Metagenomic Sequencing Library Preparation protocol (Illumina). Recommended adaptors were added to the amphibian-specific primer sequences to allow for sample-specific identification. The initial PCR was performed following the protocol listed in the *Amphibian-specific primers* section except the PCR mixture volume was doubled to 50 µL. A portion of the PCR products were used to visualized amplification bands on a electrophoresis gel and only samples with a visible band were processed further. Samples were cleaned with AMPure XP beads using a high bead to sample ratio (90µL beads per 50µL sample) to reduce the loss of the short amplicon. The amplicons were then tagged

with sample-specific indexes (Illumnia Nextera Index Kit) for each site replicate and negative control. Samples were sequenced on an Illumina NextSeq 500 (California NanoSystems Institute, UC Santa Barbara).

The resulting sequence reads were analyzed by a collaborator, Panu Somervuo (University of Helsinki). A reference database was constructed by exporting all mitochondrial sequences belong to the Amphibia taxon from NCBI and used a 100% threshold to sample sequences to the reference sequences. A detailed description of the sequence analysis can be found in Appendix 3.5. Negative controls were used to set thresholds for false positives following the methods described in Olds et al. (2016). The number of reads in the negative controls was used to determine the threshold number of reads that reduced the false positive likelihood to $p = <0.001$, for each species. Details on the negative control thresholds can be seen in Appendix 3.6.

Xenopus*-specific Detection with qPCR:*

Environmental DNA samples were analyzed with the *Xenopus*-specific primer and probe to detect *X. laevis* DNA using quantitative PCR (qPCR). Sample and control extracts were run in triplicate using a protocol modified from Secondi et al. (2016). The first run included an internal positive control (IPC) to test the sites for contaminants that might inhibit the PCR reaction. If a single sample from a site was found to be inhibited, the extracts from all three samples were processed through a OneStep PCR Inhibitor Removal Kit spin column (Zymo Research) and re-run in triplicate, including an IPC re-run.

Each sample was processed in a 25 µL mixture: 5µL of eDNA sample template and the same concentrations of primers, probe and TaqMan that was used in the tissue sample qPCR runs. Once the samples were loaded into the qPCR plate, they were moved to

the post-PCR room for the remainder of the work. Samples were assayed by qPCR (Applied Biosystems StepOnePlus) using thermal cycle of 50°C for 30 seconds, 95°C for 10 minutes, followed by 55 cycles of 95°C for 30 seconds and 51.3°C for 1 minute. Each run included dilution standards from the synthetic DNA fragment used to test the detection threshold, a positive control of *X. laevis* tissue extract, and negative controls.

A subset of positive eDNA samples and non-target amphibian tissue extracts was sequenced to confirm the presence of *X. laevis* DNA. The samples were run on standard PCR and visualized on an electrophoresis gel. The target bands approximately 80 bp long were cut from the gel, purified, and sequenced with the Sanger method (Eton Biosciences).

Results

Primer Specificity Testing

Amphibian-specific primer set:

The tissue extracts of all the amphibian species tested amplified using the amphibian-specific primer and the DNA bands were visible on the electrophoresis gels (Figure 3.2). The amphibian samples created single strong bands, indicating highly effective amplification. The DNA extract from the mice also amplified but were not as bright as the amphibian samples, indicating low but consistent amplification. The amphibian samples and mouse samples were sequenced and each species was found to have a unique DNA sequence. The sequencing results and consensus sequences can be found in Appendix 3.7. The sequences matched their target species in the NCBI database.

Xenopus*-specific primer set:*

The *X. laevis* tissue extracts displayed a single strong amplification band following standard PCR and gel electrophoresis. The other seven species of amphibians included in the study also displayed amplification bands, often more than one (Figure 3.3). Some individuals of the same species displayed different amplification band patterns. The non-*X. laevis* species' bands were typically not as strong as *X. laevis*. Samples of the amplification bands were isolated and sequenced for comparison to *X. laevis* DNA sequences. However, the quality of the Sanger sequences was low. Several of the non-*X. laevis* amphibian extracts did not sequence and the other non-target sequences did not match sequences in the NCBI database.

All *X. laevis* tissue extract samples diluted to 1ng displayed strong positives with low cycle threshold (Ct) scores in the qPCR assay, indicating early detection from a high number of copies of the targeted DNA segment (Figure 3.4). The serial dilution of *X. laevis* tissue extract found a limit of detection to be approximately 8.0^{-5}ng. The *Xenopus*-specific primer amplified *P. regilla* and *R. draytonii* tissue extracts. In addition, *A. boreas* and *T. torosa* tissue extracts displayed some amplification but fell below the 0.1 amplification threshold. While the *P. regilla* and *R. draytonii* tissue extract amplified, they displayed high Ct scores, indicating that low numbers of the targeted DNA segment were detected, despite their high DNA concentrations (50 ng). When the *P. regilla* and *R. draytonii* extracts were diluted to 5ng, they were not detected.

eDNA Samples

Amphibian-specific Detection with Next-Generation Sequencing:

Of the 15 sites assayed with the amphibian-specific primer, only nine of those sites displayed amplification in gel electrophoresis and were processed using next-generation

sequencing (NGS). Each site had three eDNA replicates but only the individual replicate samples that displayed amplification were sequenced. The amphibian-specific primer detected the presence of multiple California native and invasive amphibian species in all nine of the sites (Table 3.1).

X. laevis DNA was detected at three of the sites: Santa Clara River (site 3), Sespe Creek, and Santa Paula Creek. All of these sites were also positive for *X. laevis* using the *Xenopus*-specific primer. None of the remaining eDNA samples or negative controls contained *X. laevis* sequence reads. Several other amphibian species were detected in the samples with *X. laevis*: *L. catesbeianus*, *P. regilla*, *P. cadaverina*, and *A. boreas*. These species were also detected at multiple other sites that did not have *X. laevis*, along with other native amphibian species, *A. californicus* and *T. torosa*.

The sequence read from the eDNA NGS results (Table 3.2) were matched to their species using all amphibian genetic sequences available on the NCBI genetic database. Any sequence from the eDNA NGS results was required to match 100% to an amphibian sequence from the NCBI database to qualify as a match. Thirty-four sample negative controls, site negative controls, extract controls, and container negative controls were run in the NGS along with the field water samples. There were low levels of background contamination in all the negative controls, which tended to be, *P. regilla*, the most common species found in the field samples. The statistical analysis (Olds et al. 2016) set false-negative threshold to reduce the number of false positives. The majority of the negative controls that amplified as low positives, 54 of 63 total, were discounted based on the thresholds (Appendix 3.7, Table 1). The number of positives for *X. laevis* remained unchanged because there was no contamination of *X. laevis* DNA in the negative controls.

Xenopus-*specific Detection with qPCR:*

Fourteen of the 40 sites were found positive for *X. laevis* (Table 3.1). Thirteen of the positive responses were from sites where *X. laevis* had been found in previous occasions or was likely to be found because the creek was a tributary of a known positive river, including two locations where *X. laevis* were observed during the site visit to collect the water samples (HRNA and Piru Creek). Two positives were from sites representing new detection of *X. laevis*, Aliso Creek and Gaviota Creek but they each only had one positive detection in the nine replicates. The assay failed to detect *X. laevis* at six sites where they were likely present but were not observed when water samples were collected. The remaining 19 sites were unknown or unlikely to have *X. laevis* and *X. laevis* was not detected in the eDNA samples.

The OneStep PCR Inhibitor Removal Kit was successful in removing the inhibitors from most samples. Eighteen sites showed some inhibition in their samples prior to treatment, often only in one or two of the three sample replicates from a given site. After treatment with the inhibitor removal kit there was still some level of inhibition in six sites (Full Mill Creek, Gaviota Creek, Tijuana River, San Diego Creek, San Dieguito River, Harbison Creek) but at least 1/3 of the replicates for each of these sites were not inhibited. These sites tended to have the darkest colored extracts even after inhibitor removal.

Other amphibian species were observed at many of the eDNA sites that were *X. laevis* negative. For example, several hundred *P. regilla* tadpoles were in the water immediately above the water collection points in Carpentaria Creek; several *T. torosa* were found within a few meters above and immediately at the Tuna Creek sample collection site; two post-metamorphic *R. muscosa* were in the pool where water was collected from Full Mill Creek. *Lithobates catesbeianus* and *A. boreas* were also observed at additional sites.

The synthetic *X. laevis* fragment (gBlock) was detected at one copy per sample. There may have been some degradation of the gBlock over time because the lowest concentration standard was not consistently detected in later plates. Since the standards may be unreliable, the qPCR Ct score may be a better indicator of the level of *X. laevis* DNA present in the samples. The qPCR Ct scores for the eDNA samples and tissue extracts display a range of Ct scores (Figure 3.5).

Discussion

Environmental DNA samples successfully detected *X. laevis* and native amphibian species together at multiple sites. While only nine field sites were analyzed using the amphibian-specific primer, three sites, Santa Clara River, Sespe Creek, and Santa Paula Creek, detected *X. laevis* and several native amphibian species (Table 3.1), suggesting co-occurrence at the stream level. Both Sespe Creek and Santa Paula Creek are tributaries to the Santa Clara River and while the Santa Clara River is known to have *X. laevis* populations, this is the first record of *X. laevis* presence in Santa Paula Creek. It is unclear how far up these tributaries *X. laevis* has invaded based on only these eDNA samples. DNA has been found to travel from a few hundred meters to several kilometers in stream systems (Deiner and Altermatt 2014). It would be necessary to survey additional points up the watershed to evaluate co-existence in the upper reaches of the tributaries.

Sensitive amphibian species, *A. californicus* and *P. cadaverina*, are both present in the Santa Clara River watershed but are habitat specialists that typically occupy riparian habitat farther up tributary creeks. The habitat specializations may facilitate co-occurrence between *X. laevis* and native amphibians at the stream scale because native California

amphibians are adapted to ephemeral water habitats, while *X. laevis* is reliant on perennial water sources (Crayon 2005). Another common invasive species, *L. catesbeianus*, is also adapted to perennial water sources and research has found that naïve amphibians may be capable of co-existing with this large generalist predator because of microhabitat partitioning and reproduction advantages native amphibians have in ephemeral waters (Cook and Jennings 2007).

The other sites included in the NGS with the amphibian-specific primer (Cedar Creek, Escondido Creek, Piru Creek above Piru Lake, and Tuna Creek) were unlikely to have *X. laevis* and the sequencing results supported that hypothesis. Theses sites acted as negative controls for *X. laevis* detection and as tests to determine if a range of species could successfully be detected using the amphibian-specific primer. Both *A. californicus* and *T. torosa* were detected in these samples, further indicating that the amphibian primer can successfully amplify a diverse range of California amphibian species.

It is possible that the sample that did not display amplification with the amphibian-specific primer, and were therefore not included in the NGS, did have amphibian DNA in them but the concentrations were too low to be visible on the electrophoresis gel. For example, *X. laevis* was observed at the Hedrick Ranch Nature Area site but none of the three replicate samples appeared to amplify. In addition, *X. laevis* was detected at this site using the *Xenopus*-specific primer, indicating that amphibian DNA was present in the eDNA samples from the Hedrick Ranch Nature Area site. A better approach would have been to treat all samples as though they had amphibian DNA, even if it was not visible using electrophoresis, and process all the samples through NGS.

The eDNA negative control samples displayed low levels of contamination from several amphibian species that could not be completely eliminated, leaving some known false

positives. The contamination was typically *P. regilla* DNA but traces of other amphibian species were also found in some negative controls (Table 3.2). The contamination levels were low relative to the number of reads of *P. regilla* in eDNA water samples. While it is common to have some level of background contamination in NGS results from the common species, especially from the most common species present in the samples (Olds et al. 2016), there were still nine amphibian species "detected" in the negative controls and potentially a similar number of false positives in the site samples. It is unclear what can be done to further reduce the level of false positives given the inherent levels of contamination common in NGS (Olds et al. 2016).

The amphibian-specific primer detected several other species that are unlikely to be present at the sites. For example, *T. torosa* was not detected at any sites but its close relative, *T. granulosa*, was detected at a site where *T. torosa* were observed during the water sample collection. A similar situation occurred with a variety of toad species (i.e. *Bufo exsul* and *B. canorus*) whose DNA was detected but are not likely to be present at the sites because of their limited range; they are, however, close relatives of the *Anaxyrus* species that are likely present and were detected at multiple sites. The NGS results are already required to match amphibian sequences from the NCBI genetic database by 100% so the "detection" of these species that are not present in the sample areas are likely the result of sequencing errors, either in the NCBI database or from the eDNA NGS results. The reference database created from the amphibian tissue extracts show that only a couple of base pairs distinguish some closely related amphibian species in this short targeted DNA segment (Appendix 3.7), so errors in a few base pair could cause a match to a different species. Further analysis may be necessary to detect erroneous sequences, potentially by requiring that more than one NGS sequence read must match more than one NCBI sequence to qualify as a match. However,

this could increase the likelihood of false negative in cases where (1) few reads are found in the eDNA samples, possible because a species is rare, or (2) few sequences of a target species are available on the NCBI database.

The *Xenopus*-specific primer successfully detected *X. laevis* in southern California where the species was observed during the water sample collection and many of the areas they have been found previously. This work appears to confirm the effectiveness of the *Xenopus*-specific primer designed by Secondi et al. (2016). This primer could be a useful tool to monitor the range of *X. laevis* and evaluate the effectiveness of any eradication attempts. Invasive populations in the Santa Clara River were detected in multiple sites on the main stem of the river as well as in smaller tributary creeks where their presence had not been confirmed. Invasive populations in the Los Angeles and San Diego areas were also detected in locations where they had previously been recorded. However, six sites where *X. laevis* have been found historically did not have *X. laevis* positive samples. It is unclear if those *X. laevis* populations have been extirpated from those areas or if it is the result of a false negative.

Some samples continued to show levels of inhibition following the inhibitor removal process, which could prevent the PCR reaction from occurring properly and result in false negatives. In addition, DNA is lost through the inhibitor treatment kit (McKee et al. 2015) and any loss of DNA in eDNA samples can reduce the detection of a rare species in the system since so little DNA is likely present in the samples to begin with. Some sites, such as Tijuana River, had high Ct score, which may indicate remaining inhibition (Hartman et al. 2005). But some sites treated with the inhibitor kit were found positive for *X. laevis* using the *Xenopus*-specific primer, suggesting that enough DNA is retained for at least some level of detection. However, a handful of sites still displayed some levels of inhibition, which

likely reduced detection of any target DNA. Sample should be tested and treated for inhibitors (Goldberg et al. 2016) but the methods still appear to be imperfect.

There appear to be few false positives with the *Xenopus*-specific primer despite the fact that it was not completely specific to *X. laevis* DNA in laboratory trials with amphibian DNA extracts. Both *P. regilla* and *R. draytonii* tissue extracts amplified, which would cause false positives in the majority of the eDNA samples given the widespread abundance of *P. regilla*. However, the tissue extract from *X. laevis* amplified earlier in qPCR (Figure 3.4) and bands were brighter in the electrophoresis gels than the non-*X. laevis* amphibian species (Figure 3.3), indicating high concentrations of DNA. Only when the *X. laevis* tissue was diluted many folds did it display Ct scores that were similar to the fully concentrated non-target species. In addition, when the tissue extracts of the non-target species were diluted from 50ng to 5ng, they were not detected. This suggests that the *Xenopus*-specific primer may only partially match to other amphibian species, resulting in non-specific binding that is not as effective at producing copies of the DNA segment.

Non-specific primer binding did not appear to occur with the *Xenopus*-specific primer on eDNA samples. Many sites had other amphibians present in high numbers, as evident by direct observation and the NGS results from the amphibian-specific primer, but these sites did not result in positive detection of *X. laevis* using the *Xenopus*-specific primer. For example, the Carpentaria Creek site, which had hundreds of *P. regilla* tadpoles immediately at the site of water sample collection, was negative for *X. laevis*. The lack of amplification in eDNA samples with high *P. regilla* DNA concentrations suggests that the non-*X. laevis* amphibian DNA does not reach high enough concentrations to cause false negatives with the *Xenopus*-specific primer.

While this study found co-occurrence of native amphibians and *X. laevis* in two tributaries of the Santa Clara River, further eDNA surveying would address the level of co-occurrence. Additional eDNA surveys working up the tributaries would estimate species co-occurrence within sections of the creeks. Unfortunately, eDNA is not an accurate method to estimate species abundance (Lodge et al. 2012; Goldberg et al. 2016), so traditional survey methods would have to be used to determine if *X. laevis* affects the population sizes of native amphibians.

Environmental DNA successfully detected the presence of targeted amphibian species but there appear to be issues with false positives with the amphibian-specific primer and false negatives with the *Xenopus*-specific primer. It may be necessary to collect further eDNA samples to confirm the presence a species with low number of NGS reads, as well as sites with only a single *X. laevis* positive sample from qPCR. It may also be necessary to use other survey methods at sites that showed high levels of inhibition. Environmental DNA is a tool for detecting species that can be more efficient and effective than traditions surveys (Darling and Mahon 2011) but as with any tool, must be wielded with appropriate controls and may not be the proper survey method for all sites.

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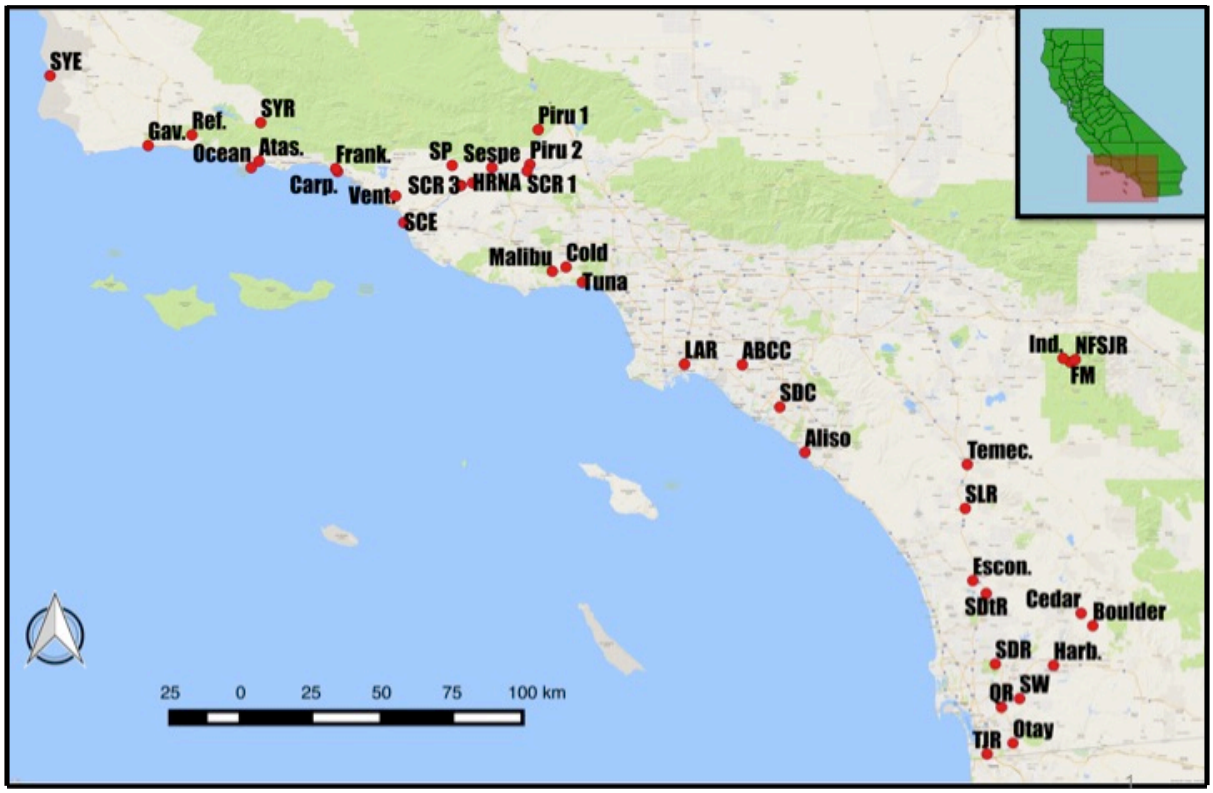
Tables & Figures



Map citation: Quantum GIS Development Team (2017), 2,12.1 Lyon. Quantum GIS. Open Source Geospatial Foundation Project. http://qgis.osgeo.org. Google Maps (2017) open source Google Streets base layer.

Figure 3.1. Map of eDNA sample locations

Map ID	Site
Aliso	Aliso Creek
ABCC	Anaheim Barbar City Channel
Atas.	Atascadero Creek
Boulder	Boulder Creek
Carp.	Carpinteria Creek
Cedar	Cedar Creek
Cold	Cold Creek
Escon.	Escondido Creek
Frank.	Franklin Creek
FM	Fuller Mill Creek
Gav.	Gaviota Creek
Harb.	Harbison Creek
HRNA	Hedrick Ranch Nature Area ditch
Ind.	Indian Creek
LAR	Los Angeles River
Malibu	Malibu Creek
Ocean	Pacific Ocean
NFSJR	North Fork San Jacinto River

Map ID	Site
Piru 2	Piru Creek below Piru Dam
QR	Quarry Road storm drain
Ref.	Refugio Creek
SDC	San Diego Creek
SDR	San Diego River
SDtR	San Dieguito River
SLR	San Luis Rey River
SCR 1	Santa Clara River 1
SCR 2	Santa Clara River 2
SCR 3	Santa Clara River 3
SCE	Santa Clara River Estuary
SP	Santa Paula Creek
SYE	Santa Ynez River Estuary
SYR	Santa Ynez River
Sespe	Sespe Creek
SW	Sweetwater River
Temec.	Temecula Creek
TJR	Tijuana River

Otay	Otay River
Piru 1	Piru Creek above Piru Dam

Tuna	Tuna Creek
Vent.	Ventura River

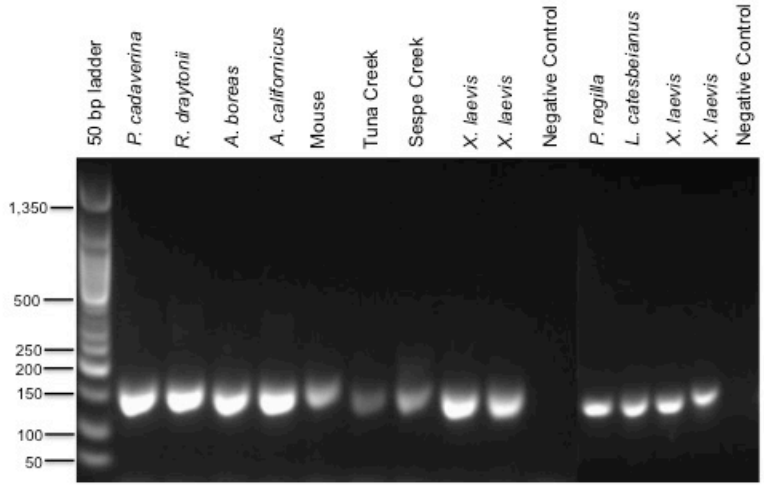


Figure 3.2. Amplicons from amphibian-specific primer
PCR products of tissue extracts and eDNA samples created with the amphibian-specific primer.

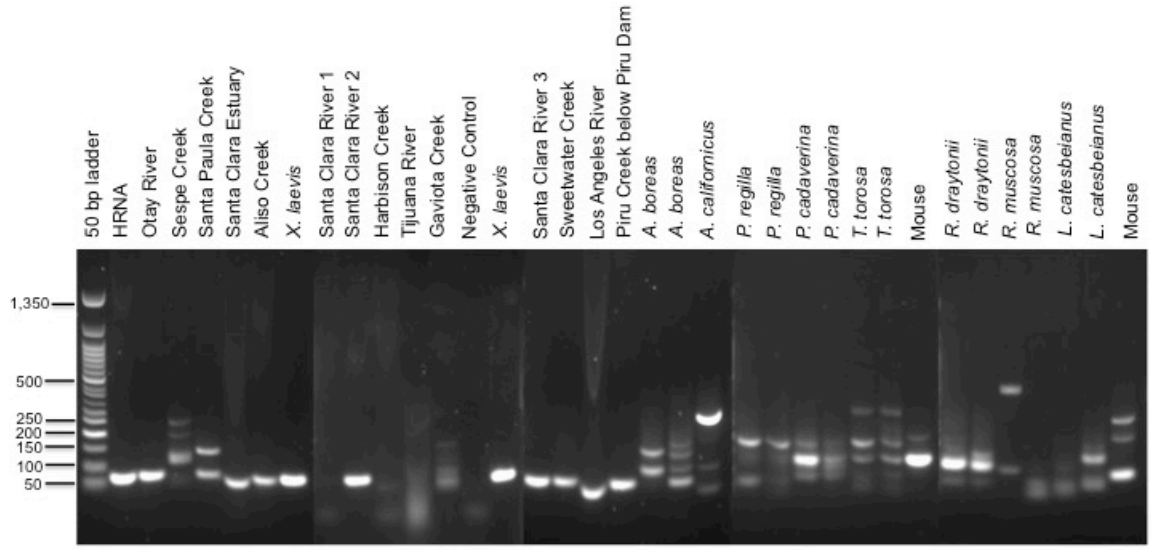


Figure 3.3. Amplicons from *Xenopus*-specific primer
PCR products of tissue extracts and eDNA samples created with the *Xenopus*-specific primer.

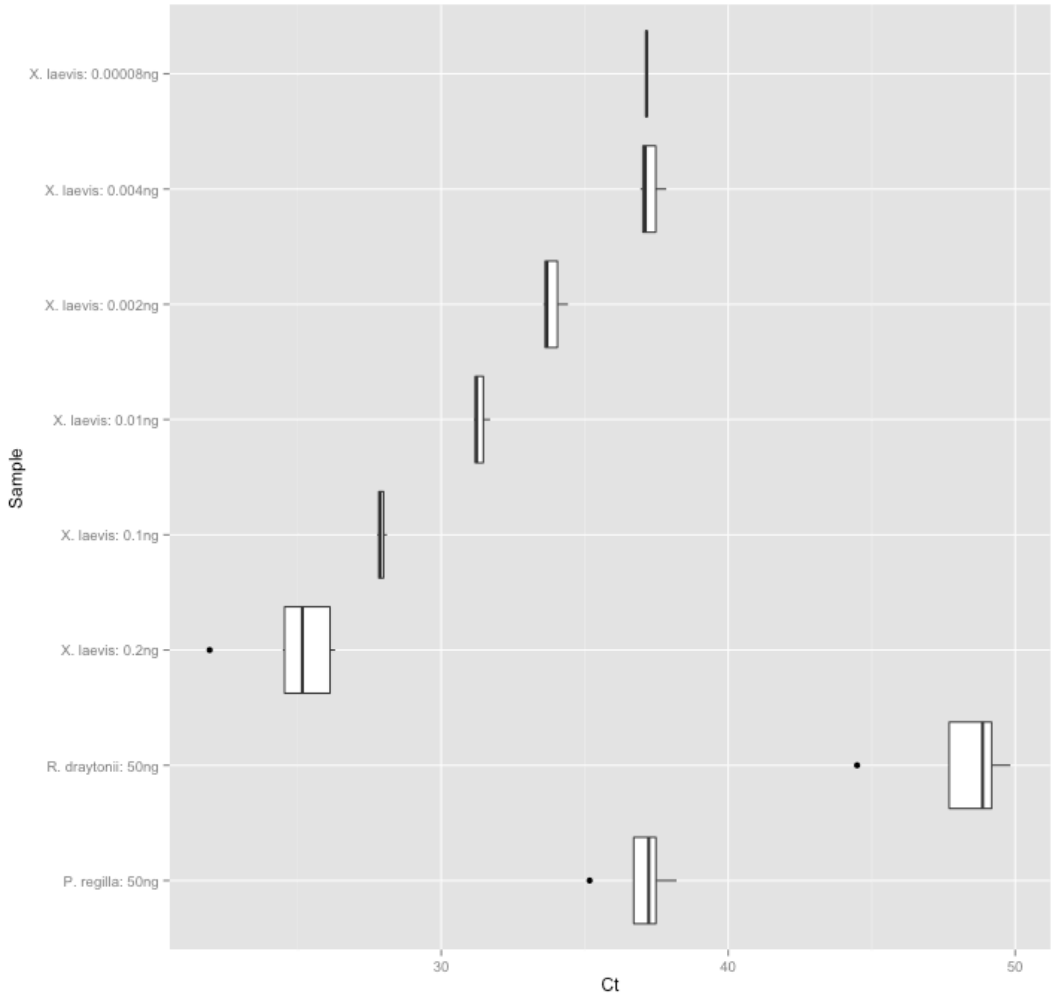


Figure 3.4. Ct scores from amphibian tissue extracts
Tissue samples from *X. laevis*, at varying DNA concentration, 50ng of *P. regilla* DNA, and 50ng of *R. draytonii* DNA were assayed with the *Xenopus*-specific primer using qPCR. The cycle threshold (Ct) scores, at which each amphibian tissue sample crossed the 0.1 PCR threshold, indicate the amount of target DNA detected.

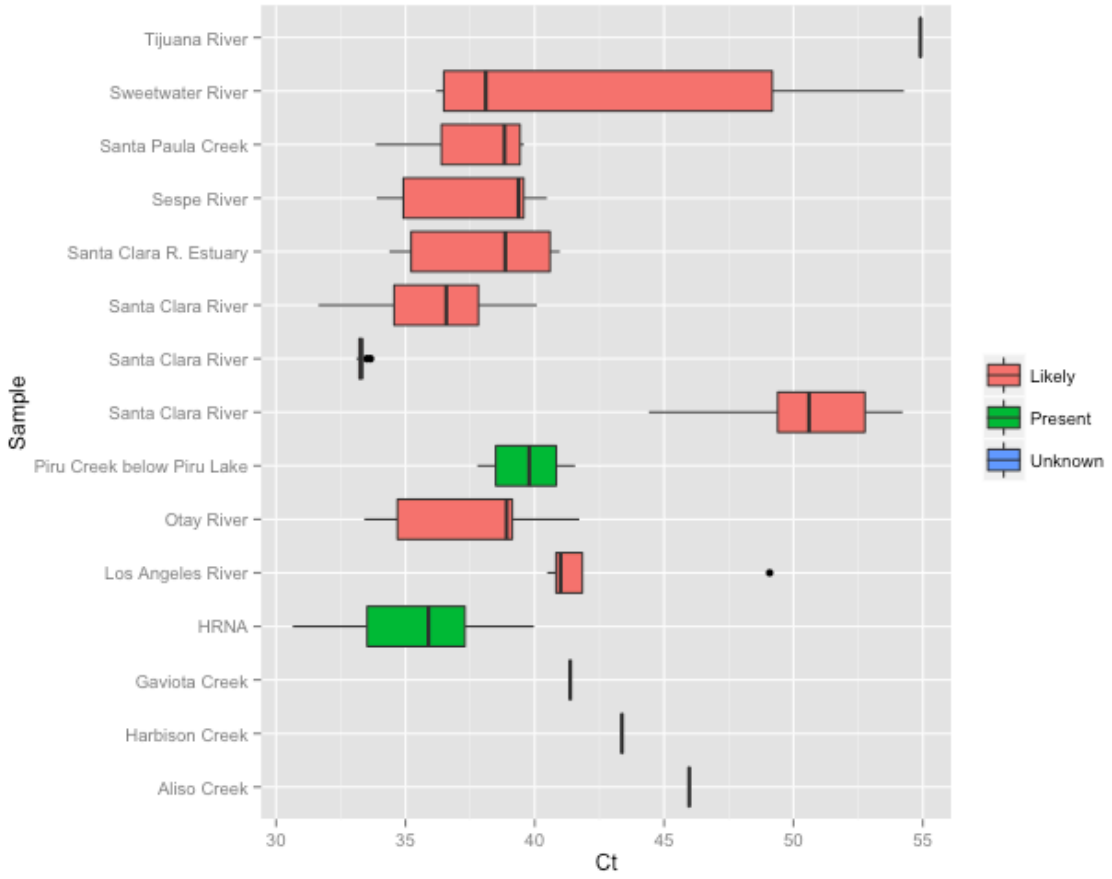


Figure 3.5. Ct scores from eDNA samples
The qPCR Ct scores for the eDNA samples positives using the *Xenopus*-specific primer. Colors distinguish likelihood that *X. laevis* based on previous collections, proximity to known invaded sites, and direct observation at the time of the sample collection. The Ct scores, at which each amphibian tissue sample crossed the 0.1 PCR threshold, indicate the strength of the detection.

Table 3.1. Detection of amphibian species using eDNA
Detection of *X. laevis* using the *Xenopus*-specific primer with qPCR and the amphibians detected using the amphibian-specific primer using next-generation sequencing.
*Species observed during site visit

		Detection with *Xenopus*-specific Primer	Detection with Amphibian-specific Primer							
Site	**Likelihood of *X. laevis* presence**	***X. laevis***	**Number of Sample Replicates Included**	***X. laevis***	***L. catesbeianus***	***P. regilla***	***P. cadaverina***	***A. boreas***	***A. californicus***	***Taricha spp.***
Aliso Creek	Likely	Detected	0	-	-	-	-	-	-	-
Anaheim Barbar City Channel	Likely	Not Detected	-	-	-	-	-	-	-	-
Atascadero Creek	Likely	Not Detected	-	-	-	-	-	-	-	-
Boulder Creek	Unlikely	Not Detected	-	-	-	-	-	-	-	-
Carpinteria Creek	Unknown	Not Detected	-	-	-	-*	-	-	-	-
Cedar Creek	Unlikely	Not Detected	3	Not Detected	Not Detected	Detected	Detected	Detected	Not Detected	Not Detected
Cold Creek	Unknown	Not Detected	-	-	-	-	-	-	-	-
Escondido Creek	Unknown	Not Detected	3	Not Detected	Not Detected*	Detected	Not Detected	Detected	Not Detected	Detected
Franklin Creek	Unknown	Not Detected	-	-	-	-	-	-	-	-
Fuller Mill Creek	Unlikely	Not Detected	-	-	-	-	-	-	-	-
Gaviota Creek	Unknown	Detected	-	-	-	-*	-	-	-	-
Harbison Creek	Unlikely	Detected	0	-	-	-	-	-	-	-

Hedrick Ranch Nature Area ditch	Present	Detected*	0	-*	-	-	-	-	-	-
Indian Creek	Unlikely	Not Detected	-	-	-	-	-	-	-	-
Los Angeles River	Likely	Detected	-	-	-	-	-	-	-	-
Malibu Creek	Unlikely	Not Detected	-	-	-	-	-	-	-	-
Collection Negative Controls	N/A	Not Detected	-	-	-	-	-	-	-	-
Pacific Ocean	Unlikely	Not Detected	-	-	-	-	-	-	-	-
North Fork San Jacinto River	Unlikely	Not Detected	-	-	-	-	-	-	-	-
Otay River	Likely	Detected	0	-	-	-	-	-	-	-
Piru Creek above Piru Dam	Unlikely	Not Detected	3	Not Detected	Detected*	Detected	Detected	Detected	Detected	Not Detected
Piru Creek below Piru Dam	Present	Detected*	-	-*	-*	-*	-	-	-	-
Quarry Road storm drain	Likely	Not Detected	-	-	-	-	-	-	-	-
Refugio Creek	Unlikely	Not Detected	-	-	-	-	-	-	-	-
San Diego Creek	Likely	Not Detected	-	-	-	-	-	-	-	-
San Diego River	Likely	Not Detected	-	-	-*	-	-	-	-	-
San Dieguito River	Likely	Not Detected	-	-	-	-	-	-	-	-
San Luis Rey River	Unlikely	Not Detected	1	Not Detected	Not Detected	Detected	Not Detected	Detected *	Not Detected	Not Detected
Santa Clara River 1	Likely	Detected	-	-	-	-	-	-*	-	-

Santa Clara River 2	Likely	Detected	-	-	-	-	-	-	-	-
Santa Clara River 3	Likely	Detected	2	Detected	Not Detected	Detected	Detected	Detected	Not Detected	Not Detected
Santa Clara River Estuary	Likely	Detected	0	-	-	-	-	-	-	-
Santa Paula Creek	Likely	Detected	3	Detected	Detected	Detected	Detected	Detected	Not Detected	Not Detected
Santa Ynez River Estuary	Unlikely	Not Detected	-	-	-	-	-	-	-	-
Santa Ynez River	Unlikely	Not Detected	0	-	-	-	-	-	-	-
Sespe Creek	Likely	Detected	3	Detected	Detected	Detected*	Detected	Detected	Not Detected	Not Detected
Sweetwater River	Likely	Detected	-	-	-	-	-	-	-	-
Temecula Creek	Unknown	Not Detected	1	Not Detected	Detected*	Detected	Not Detected	Detected	Not Detected	Not Detected
Tijuana River	Likely	Detected	-	-	-	-	-	-	-	-
Tuna Creek	Unlikely	Not Detected	3	Not Detected	Detected	Detected	Detected	Detected	Not Detected	Detected*
Ventura River	Unlikely	Not Detected	-	-	-	-*	-	-	-	-

Table 3.2. NGS results from eDNA samples
Number of next-generation sequence reads in each eDNA sample and control, by species.

		X. laevis	*L. catesbeianus*	*P. regilla*	*P. cadaverina*	*A. boreas*	*A. californicus*	*Taricha spp.*
Cedar Creek	Sample 1	0	0	872801	234143	1	0	0
	Sample 2	0	0	186486	717090	12	0	0
	Sample 3	0	0	117109	564548	0	0	0
	Negative Control	0	9	251	17	2	0	0
Escondido Creek	Sample 1	0	0	335	0	5	0	0
	Sample 2	0	0	344	0	18	0	5
	Sample 3	0	0	46	0	0	0	0
	Negative Control	0	0	68	0	8	0	0
Piru Creek above Piru Dam	Sample 1	0	495510	266534	22064	282489	22199	0
	Sample 2	0	1	28	0	0	0	0
	Sample 3	0	17194	51123	537	910	0	0
	Negative Control	0	1	2	2	0	0	0
Sespe Creek	Sample 1	0	55585	83099	18216	536840	0	0
	Sample 2	193	4759	21736	3118	151194	0	0
	Sample 3	12	9289	17640	4010	106501	0	0
	Negative Control	0	2	57	0	6	4	0
San Luis Rey River	Sample 1	0	0	75505	4	39001	0	0
	Negative Control	0	0	36	0	7	0	0
Santa Clara River 3	Sample 1	1420	0	10174	9	5562	0	0
	Sample 2	492	5	3642	22	4075	0	1
	Negative Control	0	0	77	5	1	0	0
Santa Paula Creek	Sample 1	669	26	311625	947	10	0	0
	Sample 2	906	297	324378	320	9	0	0
	Sample 3	369	2	306763	102	1	0	0
	Negative Control	0	1	28	0	11	0	0
Temecula Creek	Sample 1	0	908085	103809	0	22	0	0
	Negative Control		1	21	0	0	0	0
Tuna Creek	Sample 1	0	0	1584576	29	9	0	55340

	Sample 2	0	2	1217077	641	10	0	126253
	Sample 3	0	0	882407	11558	0	0	4472
	Negative Control	0	0	53	1	1	0	1
Aliso Creek	Negative Control	0	0	50	3	0	0	1
Harbison Creek	Negative Control	0	0	15	0	4	0	0
Hedrick Ranch Nature Area ditch	Negative Control	0	4	353	0	4	0	4
Otay River	Negative Control	0	10	0	0	0	10	
Santa Clara River Estuary	Negative Control	0	0	8	2	0	0	0
Negative Control: Ocean Water	Sample 1	0	0	3	0	0	0	0
	Sample 2	0	0	9	0	0	0	0
	Sample 3	0	0	6	0	0	0	0
	Negative Control	0	0	5	0	0	0	1
Negative Control: DNA Extraction	Extract Control 1	0	0	3	0	0	0	0
	Extract Control 2	0	0	10	1	1	0	0
Negative Control: Nalege Bottles, Jugs, & Caps	Sample a1	0	0	4	0	0	0	0
	Sample a2	0	0	4	0	0	0	0
	Sample a3	0	0	4	0	0	0	0
	Sample b1	0	0	2	0	0	0	0
	Sample b2	0	1	3	0	0	0	0
	Sample b3	0	0	2	0	0	0	0
	Sample c1	0	0	23	0	0	0	0
	Sample c2	0	6	59	0	0	0	0
	Sample c3	0	0	6	0	0	0	0
	Sample d1	0	0	4	0	0	0	0
	Sample d2	0	0	8	0	0	0	0
	Sample d3	0	0	2	0	0	0	0

Appendices

Appendix 3.1. Primer, Probe, & gBlock Sequences

Amphibian-specific (Valentini et al. 2016)**:**

Forward: 5′-ACACCGCCCGTCACCCT -3′

Reverse: 5′-GTAYACTTACCATGTTACGACTT -3′

Human DNA Blocker: 5′-TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I -3′

***Xenopus*-specific** (Secondi et al. 2016)**:**

Forward: 5′-AGGCTTAATGATTTTGCATC-3′

Reverse: 5′- AGGGTATAGAAAATGTAGCC-3′

Probe: 5′-FAM-ACGTCAGGTCAAG GTGTAGCA-BHQ1

***X. laevis* gBlock sequence:**

TGCATATCTCCGGTGCGTAACATGCAGTACAGGCTTAATGATTTTGCATCAAC
ACGTCAGGTCAAGGTGTAGCATATGAAGTGGGAAGAAATGGGCTACATTTTC
TATACCCTCACTAGCTCAGATTCAGTAGACCGCTGTTG

The same sequence with the *X. laevis* genetic sequence in capital letters and adaptor additions in lowercase letters:

tgcatatctccggtgcgtaacatgcagtacAGGCTTAATGATTTTGCATCAACACGTCAGGTCA
AGGTGTAGCATATGAAGTGGGAAGAAATGGGCTACATTTTCTATACCCTCacta
gctcagattcagtagaccgctgttg

Appendix 3.2. Environmental DNA Sample Locations

Water samples were collected from 40 sites throughout southern California and processed through a 1.2 µm pore size (47mm diameter) glass fiber filter (VWR International) (Appendix 3.2, Table 1). Three replicate samples were collected from each site and up to 2L of water was processed in each replicate. If the filter clogged from debris, less than 2L of water was processed for each filter replicate, as indicated in the "Water Volume Processed per Filter" column of Table 1.

The likelihood of *X. laevis* presence was evaluated for each field site was evaluate and given a likelihood category using data available from USGS, museum collections, and personal communications with biologists familiar with the areas. Likelihood categories were as follows:

Present: *X. laevis* were observed at the site during water sample collection

Likely: *X. laevis* have previously been collected at the site or the site is a tributary to a site of previous collection

Unknown: *X. laevis* have not been observed at the site but the site is either in highly urban area and close to "present" locations or little amphibian survey work has been conducted

Unlikely: site is geographically removed from known records of *X. laevis*, is removed from urban areas, and amphibian or other riparian work has not found *X. laevis*.

Appendix 3.2, Table 1. Sample Locations

Site	County	Latitude	Longitude	Water Volume Processed per Filter	Likelihood of *X. laevis* presence
Aliso Creek	Orange	33.512245	-117.750602	2L	Likely
Anaheim Barbar City Channel	Orange	33.788165	-117.986858	1L	Likely

Atascadero Creek	Santa Barbara	34.424738	-119.810767	2L	Likely
Boulder Creek	San Diego	32.963408	-116.664272	2L	Unlikely
Carpinteria Creek	Santa Barbara	34.39276	-119.514262	2L	Unknown
Cedar Creek	San Diego	33.002823	-116.708397	2L	Unlikely
Cold Creek	Ventura	34.094536	-118.652194	2L	Unknown
Escondido Creek	San Diego	33.106331	-117.117124	2L	Unknown
Franklin Creek	Santa Barbara	34.401589	-119.521336	2L	Unknown
Fuller Mill Creek	Riverside	33.796444	-116.749311	2L	Unlikely
Gaviota Creek	Santa Barbara	34.473601	-120.229321	2L	Unknown
Harbison Creek	San Diego	32.83743	-116.81233	0.25L	Unlikely
Hedrick Ranch Nature Area ditch	Ventura	34.357087	-119.004827	2L	Present
Indian Creek	Riverside	33.808456	-116.776425	2L	Unlikely
Los Angeles River	Los Angeles	33.790061	-118.204907	2L	Likely
Malibu Creek	Los Angeles	34.081169	-118.704012	2L	Unlikely
Pacific Ocean	Santa Barbara	34.404819	-119.840545	2L	Unlikely
North Fork San Jacinto River	Riverside	33.804217	-116.730683	2L	Unlikely
Otay River	San Diego	32.590195	-116.965602	2L	Likely
Piru Creek 1 (above Piru Dam)	Ventura	34.52292	-118.757078	2L	Unlikely
Piru Creek 2 (below Piru Dam)	Ventura	34.41483	-118.788852	2L	Present
Quarry Road storm drain	San Diego	32.705301	-117.009199	2L	Likely
Refugio Creek	Santa Barbara	34.506451	-120.064121	2L	Unlikely
San Diego Creek	Orange	33.65504	-117.845646	0.5L	Likely
San Diego River	San Diego	32.84211	-117.032327	2L	Likely
San Dieguito River	San Diego	33.065493	-117.066385	2L	Likely
San Luis Rey River	San Diego	33.335685	-117.145695	2L	Unlikely
Santa Clara River 1	Ventura	34.394641	-118.798911	2L	Likely
Santa Clara River 2	Ventura	34.349462	-119.048824	2L	Likely
Santa Clara River 3	Ventura	34.348191	-119.051141	2L	Likely
Santa Clara River Estuary	Ventura	34.233246	-119.264503	0.8L	Likely
Santa Paula Creek	Ventura	34.412008	-119.082087	2L	Likely
Santa Ynez River Estuary	Santa Barbara	34.69085	-120.599411	0.5L	Unlikely
Santa Ynez River	Santa Barbara	34.544933	-119.804429	2L	Unlikely
Sespe Creek	Ventura	34.405034	-118.931933	2L	Likely

Sweetwater River	San Diego	32.732346	-116.940646	2L	Likely
Temecula Creek	Riverside	33.474062	-117.138025	2L	Unknown
Tijuana River	San Diego	32.554547	-117.063637	2L	Likely
Tuna Creek	Los Angeles	34.046705	-118.590499	2L	Unlikely
Ventura River	Ventura	34.3164	-119.295688	2L	Unlikely

Appendix 3.3. DNA Extraction Comparison: PCI vs. Qiagen DNeasy Blood and Tissue Kit

Introduction

Environmental DNA survey methods have not yet established standard protocols for extracting DNA from filters after processing water samples. Several studies have compared DNA extraction protocols with a variety of filter types (Renshaw et al. 2014; Deiner et al. 2015; Eichmiller et al. 2015) but none have included a comparison between the two most common extraction techniques: phenol-chloroform isoamyle alcohol (PCI) and the commercially available Qiagen DNeasy Blood & Tissue Extraction Kit (Qiagen) with glass fiber filters.

Methods

I performed a controlled experiment to evaluate which DNA extraction methods would provide the most DNA from a 1.2µm, 47mm diameter, glass fiber filter using water samples from habitation tanks of *Xenopus laevis*. The *X. laevis* had been in the 12L tank for 48 hours prior to filtration of the water. Each sample filtered 0.5mL of water through the glass fiber filter. The water was stirred between before being poured into the filter funnel and the sets two sets of filter funnels were run co-currently to control for variation in water particle density from particles settling.

Three whole filters were processed by PCI and Qiagen methods. To control for variation between filters, three additional filters were cut in half and each half was processed by either PCI or Qiagen protocols.

Filters processed with the IPC method followed the protocol described in Appendix 3.4. Filters processed with the Qiagen method were processed following the recommended

protocol with the exception that the filters were initially lysed overnight while rotating at 55°C. The filters processed with the IPC method were also initially processed with this step, as well. After DNA extraction, DNA concentrations were quantified by nanodrop.

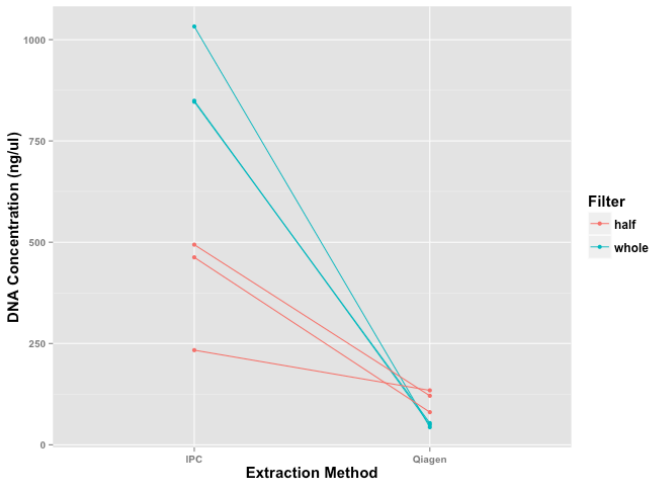
Results

Higher concentrations of DNA were obtained from PCI than Qiagen extraction method (Appendix 3.3, Table 1 & Figure 1). The differences in DNA yield between the two methods were less pronounced when DNA was extraction from the half filters but PCI still consistently resulting in higher DNA concentrations.

Conclusions

I chose to process the eDNA filters with the PCI extraction protocol over the Qiagen method because of the large differences in DNA yield. Environmental DNA samples are likely to contain only small amounts of the target DNA so it is important to maximize DNA extraction to increase the likelihood of detecting the target species.

Filter Number	Extraction Method	Filter Portion	DNA Concentration (ng/uL)
1	Qiagen	whole	43.3
3	Qiagen	whole	49.2
5	Qiagen	whole	53.6
2	IPC	whole	1032.5
4	IPC	whole	849.3
6	IPC	whole	846.5
7	Qiagen	half	134.3
7	IPC	half	233.6
8	Qiagen	half	121.0
8	IPC	half	494.0
9	Qiagen	half	80.5
9	IPC	half	462.7



Appendix 3.3, Table 1 & Figure 1. The DNA concentration extracted from each filter using the two extraction methods.

References

Deiner K, Walser J-C, Mächler E, Altermatt F (2015) Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biological Conservation 183:53–63.

Eichmiller JJ, Miller LM, Sorensen PW (2015) Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. Molecular Ecology Resources n/a–n/a. doi: 10.1111/1755-0998.12421

Renshaw MA, Olds BP, Jerde CL, et al (2014) The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. Molecular Ecology Resources 15:168–176.

Appendix 3.4. Phenol-Chloroform Isoamyl Alcohol DNA Extraction Protocol for eDNA Filters

Part 1:

1. Thaw samples in fridge.
2. Add 40 µL of Proteinase K (4mg/mL) to each vial (keep about a 700 µL:20 µL; sample:Proteinase K ratio).
3. Mix vials by inverting them several times.
4. Incubate the vials overnight at 56°C on rotator.
5. Label and UV three 1.5 mL tubes for each site.

Part 2:

- **Perform this part under fume hood with solvent resistant tips.**
- **All waste with PCI and CI must be disposed of properly.**
- **100% ethanol should be kept in freezer until use.**

1. Take all the liquid out of the 1.5 mL vial with a 1000µL pipet tip and put it into a new labeled 1.5mL vial. Remove the filter from the vial and squeeze out as much liquid as possible. If there is more than 1.5mL, you will have to divide it into two separate vials and pool it later.
2. Add enough PCI (phenol chloroform isoamyl alcohol) to double to the total volume of liquid to each vial (1 sample : 1 PCI). PCI has two layers, withdraw PCI from the bottom layer (organic phase).
3. Shake manually for 5 minutes.
4. Centrifuge for 5 minutes at 10,000 rpm.

5. Label new 1.5 mL vials for each sample. Add enough CI each vial to make a 1:1 ratio of sample:CI.
6. Collect the top layer from the sample/PCI vial and put it into the labeled 1.5 µL vial with the CI. Dump the bottom layer into the waste container.
7. Shake manually the sample/CI vial for 5 minutes.
8. Centrifuge for 5 minutes at 10,000 rpm.
9. Collect the top supernatant and place that in a new 1.5 µL vial (if you think all the Phenol is not removed, you can repeat steps 6-9). Discard the bottom layer.
10. Add ~50 µL of 5M NaCl to each vial (~10% of the sample volume).
11. Add ~1,000 µL of **ice cold** 100% EtOH to each vial (~200% of the sample volume).
12. Invert samples a few times to mix them.
13. Freeze overnight at -20°C or for a minimum of 1 hour at -80°C.

Part 3:

- **Centrifuge must be performed in the cold room at 4°C.**
- **Move centrifuge into cold room the night before use and when you move it back don't use it for several hours. All parts need to be at ambient temperature.**
- **Always load vials into the centrifuge with the hinge part out so the pellet will always be at the bottom hinge side of the vial, even if not visible.**

1. Centrifuge samples for 30 minutes at 14,000 rpm at 4°C.
2. Decant off EtOH, being careful not to take the pellet (might not be visible).
3. Add 900 µL of 70% EtOH. Pipette up and down enough times to break up and wash pellet.
4. Centrifuge for 30 minutes at 14,000 rpm at 4°C.

5. Pipette off EtOH, being careful not to take the pellet (but remove as much EtOH as possible).
6. Let the vial air dry with lid open in laminar flow hood for 15 minutes. Can put vials into 55°C hot plate for a few minutes to get last of EtOH. **ALL** EtOH must be gone!
7. Re-suspend DNA in 100 µL of 0.25X TE.
8. Place in incubator at 55°C for 10 minutes to re-dissolve DNA, remove from incubator, gently vortex and store at -20°C.

Appendix 3.5. Next-Generation Sequencing Analysis Methods

The following is a description of the analysis performed by Panu Somervuo (University of Helsinki) on the next-generation sequences results from the amphibian-specific primer.

1. Reference sequence database was constructed by exporting all mitochondrial sequences belonging to taxon Amphibia from NCBI using Entrez tools esearch and efetch (https://www.ncbi.nlm.nih.gov/books/NBK25500/). This resulted in 144828 mt sequences. Taxonomy information of each sequence was extracted from the FASTA header line.
2. Paired-end Illumina reads were assembled into single reads using pear (Zhang et al. 2014).
3. Quality filtering was applied to reads and PCR primers were removed using cutadapt (Martin 2011). Reads shorter than 40 bp were removed. Note that adapter sequences are removed during the same process since they are outside of PCR primers in the sequence construct.
4. Reads were clustered with 100% similarity threshold using usearch (Edgar 2010).
5. Cluster representative sequences were mapped against reference sequences with usearch_global (Edgar 2010).
6. Taxon count tables were constructed for each sample based on read mapping and taxonomy information of reference sequences. Cluster sizes of reads were taken into account when summarizing species abundances.

References

Edgar,RC (2010) Search and clustering orders of magnitude faster than BLAST, Bioinformatics 26(19), 2460-2461.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal, 17(1), pp. 10-12.

Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics, 30(5), 614–620.

Appendix 3.6. Thresholds for Contamination in Next-Generation Sequencing Results

Multiple negative controls were taken throughout the experiment to evaluate the potential for false positives from background contamination. Thirty-four negative control samples were run in the NGS along with the field water samples. There were low levels of background contamination in all the negative controls, which tended to be the most common species found in the field water sample replicates (Table 3.2). It follows that the higher the number of reads from a species in field sample, the more likely that sequence will be a contaminant Olds et al. (2016).

Negative controls were used to set levels of false positives following the methods described in Olds et al. (2016). The number of reads in the negative controls were used to determine the threshold number of reads that result in a false positive level <0.001, for each species (Appendix 3.6, Table 1). The samples had to fall above the threshold number of reads to be considered a positive for a given species. Several species, *X. laevis*, *R. draytonii*, and *R. muscosa*, did not contribute to the background contamination and because they did not display reads in negative controls, any sample with reads of these species was considered positive.

Using the threshold from this process, the number of false positives in negative controls was reduced from sixty-three to nine. Eleven eDNA water samples had reads below the thresholds and were therefore discounted as detections because they were likely the result of cross-contamination. The detection of a species was discounted for only three sites because other sample replicates at those sites fell above the detection threshold. Detection of *L. catesbeianus* and *T. torosa* were lost at the Santa Clara River (site 3), and *L. catesbeianus* detection was lost at the Tuna Creek site.

Species	Threshold (reads)	Percent False Positives
Xenopus laevis	0	0.0%
Lithobates catesbeianus	8	9.1%
Pseudacris regilla	101	9.1%
Pseudacris cadaverina	8	6.1%
Anaxyrus boreas	7	6.1%
Anaxyrus californicus	0	0.0%
Rana draytonii	0	0.0%
Rana muscosa	0	0.0%
Taricha spp.	3	0.0%

Appendix 3.6, Table 1. Sequence read thresholds by species

Appendix 3.7. Amphibian Reference Sequences

The following sequences were created from amphibian tissue extracts using a amphibian-specific primer (Valenitini et al. 2016). The amplicons were sequenced using Sanger methods (Eton Biosciences, San Diego) in forward and reverse (Appendix 3.7, Table 1). The primers were trimmed from the sequences and the sequences were then aligned with Geneious software (version 8.1.4) using the MUSCLE Alignment tool. If individuals of a species had a discrepancy, the base pair shared with the most number of individuals was selected.

Appendix 3.7, Table 1.

Species	Sample	Sequence	Length (bp)
Xenopus laevis	**Consensus**	**CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC**	54
	Individual 1	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
	Individual 2	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
	Individual 3	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
	Individual 4	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
	Individual 5	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
	Individual 6	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
	Inidvidual 7	CTTCTACAAAAATCAACCAATTTTATAAACACACAATTAACACAAAGAAGAGGC	54
Anaxyrus boreas	**Consensus**	**CTTCAAAGCCATCAGCCTAGTTTTTAACAACTAAGGGCGTCACAGAAGAGGC**	52
	Individual 1	CTTCAAAGCCATCAGCCTAGTTTTTAACAACTAAGGGCGTCACAGAAGAGGC	52
	Individual 2	CTTCAAAGCCATCAGCCTAGTTTTTAACAACTAAGGGCGTCACAGAAGAGGC	52
	Individual 3	CTTCAAAGCCATCAGCCTAGTTTTTAACAACTAAGGGCGTCACAGAAGAGGC	52
Anaxyrus californicus	**Individual 1**	**CTTCAAAGCTAATCTAAACTAGTTTTTAACATATTAAAGCCTTACAGAAGAGGC**	54
Lithobates catesbeianus	**Consensus**	**CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC**	
	Individual 1	CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	53
	Individual 2	CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	53
	Individual 3	CTTCGATAGTACTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	52
	Individual 4	CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	53
	Individual 5	CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	53
	Individual 6	CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	53
	Inidvidual 7	CTTCGATAGTATCTCACCCCGTTCCTAACCCACTATTACATTTTAGAAGAGGC	53

Pseudacris cadaverina	**Consensus**	**CTTCAACACCAAAAAATAGTATATAACATATCTTAGTAAATTAGAAGAGGC**	51
	Individual 1	CTTCAACACCAAAAAATAGTATATAACATATCTTAGTAAATTAGAAGAGGC	51
	Individual 2	CTTCAACACCAAAAAATAGTATATAACATATCTTAGTAAATTAGAAGAGGC	51
	Individual 3	CTTCAACACCAAAAAAATAGTATATAACATATCTTAGTAAATTAGAAGAGGC	52
	Individual 4	CTTCAACACCAAAAAATAGTATATAACATATCTTAGTAAATTAGAAGAGGC	51
	Individual 5	CTTCAACACCAAAAAATAGTATATAACATATCTTAGTAAATTAGAAGAGGC	51
Pseudacris regilla	**Consensus**	**CTTCAATCCAARAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC**	50
	Individual 1	TTCAATCCAAAAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	49
	Individual 2	CTTCAATCCAAAAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 3	CTTCAATCCAAGAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 4	CTTCAATCCAAGAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 5	CTTCAATCCAAAAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 6	CTTCAATCCAAGAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 7	CTTCAATCCAAGAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 8	CTTCAATCCAAAAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
	Individual 9	CTTCAATCCAAGAAATAGTATATAACACATATCAGTAAACTAGAAGAGGC	50
Taricha torosa	**Consensus**	**CTTCAAATCAACCACAACCCATAAATAAGAAAACCACAAAAAAAGAAGAGGC**	52
	Individual 1	CTTCAAATCAACCACAACCCATAAATAAGAAAACTACAAAAAAAGAAGAGGC	52
	Individual 2	CTTCAAATCAACCACAACCCATAAATAAGAAAACCACAAAAAAAGAAGAGGC	52
	Individual 3	CTTCAAATCAACCACAACCCATAAATAAGAAAACCACAAAAAAAGAAGAGGC	52
	Individual 4	CTTCAAATCAACCACAACCCATAAATAAGAAAACCACAAAAAAAGAAGAGGC	52
	Individual 5	CTTCAAATCAACCACAACCCATAAATAAGAAAACCACAAAAAAAGAAGAGGC	52
	Individual 6	CTTCAAATCAACCACAACCCATAAATAAGAAAACCACAAAAAAAGAAGAGGC	52
Rana draytonii	**Consensus**	**CTTCAATAGTATTTTCCTGTCCCTAACCACCACACACATTTTAGAAGAGGC**	51
	Individual 1	CTTCAATAGTATTTTCCTGTCCCTAACCACCACACACATTTTAGAAGAGGC	51
	Individual 2	CTTCAATAGTATTTTCCTGTCCCTAACCACCACACACATTTTAGAAGAGGC	51
	Individual 3	CTTCAATAGTATTTTCCTGTCCCTAACCACCACACACATTTTAGAAGAGGC	51